

Article 34

**PROTEINS WITH ENHANCED LEVELS
OF ESSENTIAL AMINO ACIDS**

Field of the Invention

5 The present invention relates to the field of protein engineering wherein changing amino acid compositions effects improvements in the nutrition content of feed. Specifically, the present invention relates to methods of enhancing the nutritional content of animal feed by expressing derivatives of a protease inhibitor to provide higher percentages of essential amino acids in plants.

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Background of the Invention

Feed formulations are required to provide animals essential nutrients critical to growth. However, crop plants are generally rendered food sources of poor nutritional quality because they contain low proportions of several amino acids which are essential
15 for, but cannot be synthesized by, monogastric animals.

For many years researchers have attempted to improve the balance of essential amino acids in the seed proteins of important crops through breeding programs. As more becomes known about seed storage proteins and the expression of the genes which encode these proteins, and as transformation systems are developed for a greater variety of plants,
20 molecular approaches for improving the nutritional quality of seed proteins can provide alternatives to the more conventional approaches. Thus, specific amino acid levels can be enhanced in a given crop via biotechnology.

One alternative method is to express a heterologous protein of favorable amino acid composition at levels sufficient to obviate feed supplementation. For example, a
25 number of seed proteins rich in sulfur amino acids have been identified. A key to good expression of such proteins involves efficient expression cassettes with tissue-preferred promoters. Not only must the gene-controlling regions direct the synthesis of high levels of mRNA, the mRNA must be translated into a stable protein and over expression of this protein must not be detrimental to plant or animal health.

30 Among the essential amino acids needed for animal nutrition, often limiting in crop plants, are methionine, threonine, lysine, isoleucine, leucine, valine, tryptophan, phenylalanine, and histidine. Attempts to increase the levels of these free amino acids by

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breeding, mutant selection and/or changing the composition of the storage proteins accumulated in crop plants has met with limited success.

A transgenic example is the phaseolin-promoted Brazil nut 2S expression cassette. However, even though Brazil nut protein increases the amount of total methionine and bound methionine, thereby improving nutritional value, there appears to be a threshold
5 limitation as to the total amount of methionine that is accumulated in the seeds. The seeds remain insufficient as sources of methionine and methionine supplementation is required in diets utilizing the above soybeans.

An alternative to the enhancement of specific amino acid levels by altering the
10 levels of proteins containing the desired amino acid is modification of amino acid biosynthesis. Recombinant DNA and gene transfer technologies have been applied to alter enzyme activity catalyzing key steps in the amino acid biosynthetic pathway. See Glassman, U.S. Patent No. 5,258,300; Galili, et al., European Patent Application No. 485970; (1992); incorporated herein in its entirety. However, modification of the amino
15 acid levels in seeds is not always correlated with changes in the level of proteins that incorporate those amino acids. See Burrow, et al., Mol. Gen. Genet.; Vol. 241; pp. 431-439; (1993); incorporated herein in its entirety by reference. Increases in free lysine levels in leaves and seeds have been obtained by selection for DHDPS mutants or by expressing the E. coli DHDPS in plants. However, since the level of free amino acids in
20 seeds, in general, is only a minor fraction of the total amino acid content, these increases have been insufficient to significantly increase the total amino acid content of seed.

The lysC gene is a mutant bacterial aspartate kinase which is desensitized to feedback inhibition by lysine and threonine. Expression of this gene results in an increase in the level of lysine and threonine biosynthesis. However, expression of this gene with
25 seed-specific expression cassettes has resulted in only a 6-7% increase in the level of total threonine or lysine in the seed. See Karchi, et al., The Plant J.; Vol. 3; pp. 721-7; (1993); incorporated herein in its entirety by reference. Thus, there is minimal impact on the nutritional value of seeds, and supplementation with essential amino acids is still required.

30 In another study (Falco et al., Biotechnology 13:577-582, 1995), manipulation of bacterial DHDPS and aspartate kinase did result in useful increases in free lysine and total

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seed lysine. However, abnormal accumulation of lysine catabolites was also observed suggesting that the free lysine pool was subject to catabolism.

Based on the foregoing, there exists a need for methods of increasing the levels of essential amino acids in seeds of plants. As can be seen from the prior art, previous approaches have led to insufficient increases in the levels of both free and bound amino acids and insignificant enhancement of the nutritional content of the feed.

Summary of the Invention

It is one object of the present invention to provide nucleic acids encoding protease inhibitors with modified levels of essential amino acids. It is an object to reduce the protease inhibitory activity in addition to modifying levels of essential amino acids and antigenic polypeptide fragments thereof. It is a further object of the present invention to provide transgenic plants comprising protease inhibitors with modified levels of essential amino acids. Additionally, it is an object of the present invention to provide methods for increasing the nutritional value of a plant and for providing an animal feed composition comprising the transgenic plants comprising protease inhibitors with modified levels of essential amino acids and reduced protease inhibitory activity. The protease inhibitor CI-2 has been modified to produce on 83 amino acid polypeptide and an amino-terminal truncated version of 65 amino acids residues.

Therefore, in one aspect, the present invention relates to a polypeptide comprising at least 10 contiguous amino acid residues from a protein having Seq. ID No. 2, 4, 6, 8, 10 or 12, 16, 18, 20, 22, 24; and wherein the polypeptide exhibits reduced protease inhibitor activity compared to a wild-type protein. In one embodiment, the present invention relates to the above mentioned polypeptide comprising Seq. ID No. 2, 4, 6, 8, 10 or 12, 16, 18, 20, 22, 24 and the polypeptide wherein more than about 55%, but less than about 95%, more than about 55%, but less than about 90%, or more than about 55% but less than about 85%, of the amino acid residues are essential amino acids. In some embodiments, the essential amino acid is lysine, tryptophan, methionine, threonine or mixtures thereof. In some embodiments, the present invention relates to the nucleic acid encoding the polypeptide referred to *supra* and in one embodiment, relates to the nucleic acid as DNA and in another embodiment to a second nucleic acid which is complementary to the DNA. Another embodiment relates to the polypeptide wherein more than about 10% but less than about 40% of the amino acid residues are essential

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60 61 62 63 64 65 66 67 68 69 70 71 72 73 74 75 76 77 78 79 80 81 82 83 84 85 86 87 88 89 90 91 92 93 94 95 96 97 98 99 100

amino acids. Another embodiment relates to the transformed plant containing the polypeptide *supra*. In some embodiments an animal feed composition is provided.

In another embodiment, the polypeptide referred to *supra*, comprises at least 20 contiguous amino acid residues. In one aspect, the present invention relates to this
5 polypeptide which contains or is modified to contain essential amino acids at positions 1, 8, 11, 17, 19, 34, 41, 56, 59, 62, 65, 67 or 73. In another aspect, the present invention relates to polypeptide which contains or is modified to contain essential amino acids at positions 1,16,23,41,44,49 and 55. In other embodiments, the polypeptide comprises at least 30 contiguous amino acid residues.

10 In a further aspect, the present invention relates to the modification of amino acid residues in the active site of protease inhibitors. The above mentioned polypeptide contains, or is modified to contain, non-wild type amino acid residues at positions from about 53 to about 70. In some embodiments, the non-wild type amino acid residues are located at positions 58-60, 62, 65, or 67. In another embodiment, the polypeptide the non-
15 wild type amino acid residue is located at position 59. In some embodiments, the present invention relates to the nucleic acid encoding the polypeptide referred to *supra*.

In another aspect the polypeptide is about 7.3 Kda or about 9.2 Kda and further comprises one or more additional amino terminal amino acid residues, and in some embodiments, the amino-terminal amino acid residue is methionine. In another
20 embodiment, the polypeptide is a cleavage product and in yet another, the polypeptide is recombinantly produced.

In a further aspect, the present invention relates to an expression cassette comprising the nucleic acids as described *supra*, operably linked to a promoter providing for protein expression. In some embodiments, the promoter provides for protein
25 expression in plants and in others the promoter provides for protein expression in bacteria, yeast or virus.

In yet another aspect, the present invention is directed to transformed plant cells containing the expression cassette described *supra*.

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In another aspect, the present invention is directed to transformed plants containing at least one copy of the expression cassette described *supra*. In some embodiments, there is a seed of this transformed plant.

Another aspect of this invention provides a polypeptide produced by substituting
5 an essential amino acid for at least one but less than 50 amino acid residues in a protease inhibitor for enhancing nutritional value of feed.

In another aspect, the present invention relates to polypeptides *supra* wherein hydrogen bonding is disrupted in the active site loop of the inhibitor.

In yet another aspect, the present invention relates to the polypeptide *supra* which exhibits
10 decreased protease inhibitor activity as compared to the wild-type protein which does not have substituted amino acid residues. In some embodiments nucleic acid encodes a protease inhibitor protein with decreased inhibitory activity.

In another aspect, the present invention relates to the polypeptide *supra* which exhibits less than about 30% of the inhibitor activity compared to corresponding wild-
15 type protein which does not have substituted amino acid residues.

In another aspect, the present invention relates to a nucleic acid comprising the sequence of SEQ ID No. 1,3,5,7,9,11,15,17,19,21, or 23 or a nucleic acid having at least 70% identity thereto, wherein the nucleic acid encodes for a polypeptide which exhibits reduced protease inhibitor activity compared to a wild type protein. In one embodiment,
20 the polypeptide exhibits 80% identity and in another embodiment, 90%.

In yet another aspect, the present invention relates to a nucleic acid encoding a protease inhibitor protein wherein nucleotides have been substituted to increase the number of essential amino acids in the encoded protein. In one embodiment, the inhibitor protein is derived from a plant. In another embodiment, the inhibitor protein is a
25 chymotrypsin inhibitor- like protein.

In another aspect, the present invention relates to an expression cassette comprising the nucleic acid encoding the polypeptide *supra*, operably linked to a promoter providing for protein expression. In some embodiments, the promoter provides

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for protein expression in plants. In some embodiments, the promoter provides for protein expression in bacteria, yeast or virus.

In yet another aspect, the transformed plant containing at least one copy of the expression cassette *supra*. In some embodiments, the transformed plant is a
5 monocotyledonous plant and could be selected from the group consisting of maize, sorghum, wheat, rice and barley. In some embodiments, the transformed plant is a dicotyledonous plant and could be selected from the group consisting of soybean, alfalfa, canola, sunflower, tobacco, tomato and canola. Preferably, the transformed plant is maize or soybeans. In some embodiments seed is produced by the transformed plant. In some
10 embodiments an animal feed composition is provided, and in some, the animal feed composition is the seed.

In another aspect, the present invention relates to transformed plant cells containing the expression cassette *supra*.

In another aspect, the present invention relates to a method for increasing the
15 nutritional value of a plant comprising introducing into the cells of the plant the expression cassette *supra* to yield transformed plant cells and regenerating a transformed plant from the transformed plant cells.

The present invention provides a method for genetically modifying protease inhibitors to increase the level of at least, but not limited to one, essential amino acid in a
20 plant so as to enhance the nutritional value of the plant. The methods comprise the introduction of an expression cassette into regenerable plant cells to yield transformed plant cells. The expression cassette comprises a nucleotide encoding a protease inhibitor operably linked to a promoter functional in plant cells.

A fertile transgenic plant is regenerated from the transformed cells, and seeds are
25 isolated from the plant. The seeds comprise the polypeptide which is encoded by the DNA segment and which is produced in an amount sufficient to increase the amount of the essential amino acid in the seeds of the transformed plants, relative to the amount of the essential amino acid in the seeds of a corresponding untransformed plant, e.g., the seeds of a regenerated control plant that is not transformed or corresponding
30 untransformed seeds isolated from the transformed plant.

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Preferably, the substantiated amino acid is an essential amino acid. More preferably, tryptophan, threonine, methionine and lysine are the substituted essential amino acid. Even more preferably, the additional essential amino acid is lysine.

5 A preferred embodiment of the present invention is the introduction of an expression cassette into regenerable plant cells. Also preferred is the introduction of an expression cassette comprising a DNA segment encoding an endogenous or modified polypeptide sequence.

The present invention also encompasses variations in the sequences described above, wherein such variations are due to site-directed mutagenesis, or other mechanisms
10 known in the art, to increase or decrease levels of selected amino acids of interest. For example, site-directed mutagenesis to increase levels of essential amino acids is a preferred embodiment.

The present invention also provides a fertile transgenic plant. The fertile transgenic plant contains an isolated DNA segment comprising a promoter and encoding a
15 protein comprising a protease inhibitor, modified by increasing the number of essential amino acids, under the control of the promoter. The protease inhibitor is expressed as so that the level of essential amino acids in the seeds of the transgenic plant is increased above the level in the seeds of a plant which only differ from the seeds of the transgenic plant in that the DNA segment or the encoded seed protein is under the control of a
20 different promoter. The DNA segment is transmitted through a complete normal sexual cycle of the transgenic plant to the next generation. The present invention provides nucleotide sequences encoding proteins containing higher levels of essential amino acids by the substitution of one or more of the amino acid residues in the protease inhibitor. Substitutions at one or more of, but not limited to, positions
25 1,8,11,17,19,34,41,56,59,62,67 and 73 of the wild type protein are substituted with essential amino acids. The present invention also involves the expression of the present chymotrypsin inhibitor derivatives or any derived protease inhibitor in plants to provide higher percentages of essential amino acids in plants than wild type plants.

In a preferred embodiment of the present invention, the present derivatives also
30 exhibit reduced protease inhibitor activity. This is achieved by substituting the amino acid residues from about amino acid residue 53 to about amino acid residue 70 with residues other than the wild type residues.

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Methods for expressing the modified protease inhibitors and for using plants are also provided to enhance the nutritional value of animal feed.

It is therefore an object of the present invention to provide methods for increasing the levels of the essential amino acids in the seeds of plants used for animal feed.

5 It is a further object of the present invention to provide seeds for food and/or feed with higher levels of the essential amino acid, lysine, than wild type species of the same seeds.

It is a further object of the present invention to provide seeds for food and/or feed such that the level of the essential amino acids is increased such that the need for feed
10 supplementation is greatly reduced or obviated.

It is one object of the present invention to provide nucleic acids encoding enzymes involved in protease inhibition and antigenic polypeptide fragments thereof. It is also an object of the present invention to provide protease inhibitor polypeptides and antigenic fragments thereof. It is a further object of the present invention to provide transgenic
15 plants comprising protease inhibitor nucleic acids. Additionally, it is an object of the present invention to provide methods for modulating, in a transgenic plant, the expression of protease inhibitor polynucleotides of the present invention.

Therefore, in one aspect, the present invention relates to an isolated nucleic acid comprising a member selected from the group consisting of (a) a polynucleotide having at
20 least 70% identity to a polynucleotide encoding a polypeptide selected from the group consisting of SEQ ID NOS: 2,4,6,8,10 and 12,16,18,20,22,24; and (b) a polynucleotide which is complementary to the polynucleotide of (a); and (c) a polynucleotide comprising at least 30 contiguous nucleotides from a polynucleotide of (a) or (b). In some embodiments, the polynucleotide has a sequence selected from the group consisting of
25 SEQ ID NOS: 1,3,5,7,9 and 11, 15,17,19,21, or 23 . The isolated nucleic acid can be DNA.

In another aspect, the present invention relates to recombinant expression cassettes, comprising a nucleic acid as described, *supra*, operably linked to a promoter. In some embodiments, the nucleic acid is operably linked in antisense orientation to the
30 promoter.

In another aspect, the present invention is directed to a host cell transfected with the recombinant expression cassette as described, *supra*. In some embodiments, the host

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cell is a maize, rye, barley, wheat, sorghum, oats, millet, rice, triticale, sunflower, alfalfa, rapeseed or soybean cell.

In a further aspect, the present invention relates to an isolated protein comprising a polypeptide of at least 10 contiguous amino acids encoded by the isolated nucleic acid referred to, *supra*. In some embodiments, the polypeptide has a sequence selected from the group consisting of SEQ ID NOS: 2,4,6,8,10 and 12,16,18,20,22,24.

In another aspect, the present invention relates to an isolated nucleic acid comprising a polynucleotide of at least 30 nucleotides in length which selectively hybridizes under stringent conditions to a nucleic acid selected from the group consisting of SEQ ID NOS: 1,3,5,7,9 and 11, 15,17,19,21, 23 or a complement thereof. In some embodiments, the isolated nucleic acid is operably linked to a promoter.

In yet another aspect, the present invention relates to an isolated nucleic acid comprising a polynucleotide, the polynucleotide having at least 60% sequence identity to an identical length of a nucleic acid selected from the group consisting of SEQ ID NOS: 1,3,5,7,9 and 11, 15,17,19,21, 23 or a complement thereof.

In another aspect, the present invention relates to an isolated nucleic acid comprising a polynucleotide having a sequence of a nucleic acid amplified from a *Zea mays* nucleic acid library using the primers selected from the group consisting of: SEQ ID NOS: 25 and 26 or complements thereof. In some embodiments, the nucleic acid library is a cDNA library.

In another aspect, the present invention relates to a recombinant expression cassette comprising a nucleic acid amplified from a library as referred to *supra*, wherein the nucleic acid is operably linked to a promoter. In some embodiments, the present invention relates to a host cell transfected with this recombinant expression cassette. In some embodiments, the present invention relates to a protease inhibitor protein produced from this host cell.

In a further aspect, the present invention relates to a heterologous promoter operably linked to a non-isolated protease inhibitor polynucleotide encoding a polypeptide, wherein the polypeptide is encoded by a nucleic acid amplified from a nucleic acid library as referred to, *supra*.

In yet another aspect, the present invention relates to a transgenic plant comprising a recombinant expression cassette comprising a plant promoter operably linked to any of

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the isolated nucleic acids referred to *supra*. In some embodiments, the transgenic plant is *Zea mays*. The present invention also provides transgenic seed from the transgenic plant.

- In a further aspect, the present invention relates to a method of providing a modified protease inhibitor in a plant, comprising the steps of (a) transforming a plant cell
- 5 with a recombinant expression cassette comprising a protease inhibitor polynucleotide operably linked to a promoter; (b) growing the plant cell under plant growing conditions; and
- (c) inducing expression of the polynucleotide .

DETAILED DESCRIPTION

Figure listing

Figure 1 Protease Inhibition

5 Sequence identification

Barley High Lysine 1(BHL-1) is coded for by the polypeptides of SEQ ID No. 2 which is encoded for by the nucleic acid of SEQ ID No. 1.

Barley High Lysine 2 (BHL-2) is coded for by the polypeptides of SEQ ID No. 4 which is encoded for by the nucleic acid of SEQ ID No. 3.

10 Barley High Lysine 3 (BHL-3) is coded for by the polypeptides of SEQ ID No. 6 which is encoded for by the nucleic acid of SEQ ID No. 5.

Barley High Lysine 3N (BHL-3N) is coded for by the polypeptides of SEQ ID No. 8 which is encoded for by the nucleic acid of SEQ ID No. 7.

15 Barley High Lysine 1N (BHL-1N) is coded for by the polypeptides of SEQ ID No. 10 which is encoded for by the nucleic acid of SEQ ID No. 9.

Barley High Lysine 2N (BHL-2N) is coded for by the polypeptides of SEQ ID No. 12 which is encoded for by the nucleic acid of SEQ ID No. 11.

20 Wild-type chymotrypsin inhibitor (WI-CI-2) is coded for by the polypeptides of SEQ ID No. 14 which is encoded for by the nucleic acid of SEQ ID No. 13.

Maize EST PI-1 is coded for by the polypeptides of SEQ ID No.16 which is encoded for by the nucleic acid of SEQ ID No. 15.

Maize EST PI-2 is coded for by the polypeptides of SEQ ID No.18 which is encoded for by the nucleic acid of SEQ ID No. 17.

25 Maize EST PI-3 is coded for by the polypeptides of SEQ ID No.20 which is encoded for by the nucleic acid of SEQ ID No. 19.

Maize EST PI-4 is coded for by the polypeptides of SEQ ID No.22 which is encoded for by the nucleic acid of SEQ ID No. 21.

30 Maize EST PI-5is coded for by the polypeptides of SEQ ID No. 24 which is encoded for by the nucleic acid of SEQ ID No. 23.

The 5' and 3' PCR primer pairs A & B, are identified as SEQ ID Nos. 25 and 26, respectively.

Definitions

Units, prefixes, and symbols may be denoted in their SI accepted form. Unless
5 otherwise indicated, nucleic acids are written left to right in 5' to 3' orientation; amino
acid sequences are written left to right in amino to carboxy orientation, respectively.
Numeric ranges are inclusive of the numbers defining the range. Amino acids may be
referred to herein by either their commonly known three letter symbols or by the one-
letter symbols recommended by the IUPAC-IUB Biochemical Nomenclature
10 Commission. Nucleotides, likewise, may be referred to by their commonly accepted
single-letter codes. The terms defined below are more fully defined by reference to the
specification as a whole.

“Chymotrypsin inhibitor-like” protein is a protein with a sequence identity of 40%
or more to the CI-2 from barley.

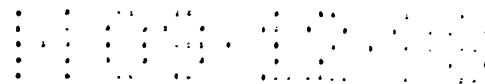
15 “%” refers to molar % unless otherwise specified or implied.

“Essential amino acids” are amino acids that must be obtained from an external
source because they are not synthesized by the individual. They are comprised of:
methionine, threonine, lysine, isoleucine, leucine, valine, tryptophan, phenylalanine, and
histidine.

20 By “amplified” is meant the construction of multiple copies of a nucleic
acid sequence or multiple copies complementary to the nucleic acid sequence using at
least one of the nucleic acid sequences as a template. Amplification systems include the
polymerase chain reaction (PCR) system, ligase chain reaction (LCR) system, nucleic
acid sequence based amplification (NASBA, Cangene, Mississauga, Ontario), Q-Beta
25 Replicase systems, transcription-based amplification system (TAS), and strand
displacement amplification (SDA). See, e.g., *Diagnostic Molecular Microbiology:
Principles and Applications*, D. H. Persing *et al.*, Ed., American Society for
Microbiology, Washington, D.C. (1993).

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As used herein, “antisense orientation” includes reference to a duplex
polynucleotide sequence which is operably linked to a promoter in an orientation where



the antisense strand is transcribed. The antisense strand is sufficiently complementary to an endogenous transcription product such that translation of the endogenous transcription product is often inhibited.

As used herein, "chromosomal region" includes reference to a length of
5 chromosome which may be measured by reference to the linear segment of DNA which it comprises. The chromosomal region can be defined by reference to two unique DNA sequences, i.e., markers.

The term "conservatively modified variants" applies to both amino acid and nucleic acid sequences. With respect to particular nucleic acid sequences, conservatively
10 modified variants refers to those nucleic acids which encode identical or essentially identical amino acid sequences, or where the nucleic acid does not encode an amino acid sequence, to essentially identical sequences. Because of the degeneracy of the genetic code, a large number of functionally identical nucleic acids encode any given protein. For instance, the codons GCA, GCC, GCG and GCU all encode the amino acid alanine.
15 Thus, at every position where an alanine is specified by a codon, the codon can be altered to any of the corresponding codons described without altering the encoded polypeptide. Such nucleic acid variations are "silent variations" and represent one species of conservatively modified variation. Every nucleic acid sequence herein which encodes a polypeptide also describes every possible silent variation of the nucleic acid. One of
20 ordinary skill will recognize that each codon in a nucleic acid (except AUG, which is ordinarily the only codon for methionine, and TGG, which is ordinarily the only codon for tryptophan) can be modified to yield a functionally identical molecule. Accordingly, each silent variation of a nucleic acid which encodes a polypeptide of the present invention is implicit in each described polypeptide sequence and incorporated herein by
25 reference.

As to amino acid sequences, one of skill will recognize that individual substitutions, deletions or additions to a nucleic acid, peptide, polypeptide, or protein sequence which alters, adds or deletes a single amino acid or a small percentage of amino acids in the encoded sequence is a "conservatively modified variant" where the alteration
30 results in the substitution of an amino acid with a chemically similar amino acid. Thus, any number of amino acid residues selected from the group of integers consisting of from 1 to 15 can be so altered. Thus, for example, 1, 2, 3, 4, 5, 7, or 10 alterations can be



made. Conservatively modified variants typically provide similar biological activity as the unmodified polypeptide sequence from which they are derived. For example, substrate specificity, enzyme activity, or ligand/receptor binding is generally at least 30%, 40%, 50%, 60%, 70%, 80%, or 90% of the native protein for its native substrate.

- 5 Conservative substitution tables providing functionally similar amino acids are well known in the art.

The following six groups each contain amino acids that are conservative substitutions for one another:

- 1) Alanine (A), Serine (S), Threonine (T);
- 10 2) Aspartic acid (D), Glutamic acid (E);
- 3) Asparagine (N), Glutamine (Q);
- 4) Arginine (R), Lysine (K);
- 5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V); and
- 6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W).

- 15 See also, Creighton (1984) Proteins W.H. Freeman and Company.

By "encoding" or "encoded", with respect to a specified nucleic acid, is meant comprising the information for translation into the specified protein. A nucleic acid encoding a protein may comprise non-translated sequence (e.g., introns) within translated regions of the nucleic acid, or may lack such intervening non-translated sequences (e.g.,
 20 as in cDNA). The information by which a protein is encoded is specified by the use of codons. Typically, the amino acid sequence is encoded by the nucleic acid using the "universal" genetic code. However, variants of the universal code, such as is present in some plant, animal, and fungal mitochondria, the bacterium *Mycoplasma capricolum* (Proc. Natl. Acad. Sci. (USA), 82:2306-2309 (1985)), or the ciliate *Macronucleus*, may
 25 be used when the nucleic acid is expressed using these organisms.

When the nucleic acid is prepared or altered synthetically, advantage can be taken of known codon preferences of the intended host where the nucleic acid is to be expressed. For example, although nucleic acid sequences of the present invention may be expressed in both monocotyledonous and dicotyledonous plant species, sequences can be
 30 modified to account for the specific codon preferences and GC content preferences of monocotyledons or dicotyledons as these preferences have been shown to differ (Murray *et al.* Nucl. Acids Res. 17: 477-498 (1989)). Thus, the maize preferred codon for a

quences from maize. Maize

Table 4 of Murray *et al.* *supra*

reference to a protease inhibitor amino acid sequence of, a native protein involved in protease size comparison relative to a particular protease inhibitor nucleic acids full-length are well known for western blots. See, e.g., *ibid.*, Springer-Verlag, Berlin. Consensus sequences can also be used to identify, additionally, consensus regions of mRNA aid in the identification of, the consensus sequence of the N-terminal methionine, aids in the identification of the 5' end. Consensus sequences are used to determine whether the

Nucleic acid is a nucleic acid that
 is, is substantially modified
 For example, a promoter
 species different from that
 same species, one or both are
 homologous protein may originate
 substantially modified from its

or and supports the replication
be prokaryotic cells such as *E.*
mammalian cells. Preferably,
cells. A particularly preferred

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The term "hybridization complex" includes reference to a duplex nucleic acid sequence formed by two single-stranded nucleic acid sequences which selectively hybridize with each other.

The terms "isolated" or "biologically pure" refer to material which is: (1) substantially or essentially free from components which normally accompany or interact with it as found in its naturally occurring environment. The isolated material optionally comprises material not found with the material in its natural environment. (2) If the material is in its natural environment, the material has been synthetically (non-naturally) altered to a composition and/or placed at a locus in the cell (e.g., genome) not native to a material found in that environment. The alteration to yield the synthetic material can be performed on the material within or removed from its natural state. For example, a naturally occurring nucleic acid becomes an isolated nucleic acid if it is altered, or if it is transcribed from DNA which is altered, by non-natural, synthetic (i.e., "man-made") methods performed within the cell from which it originates. See, e.g., Compounds and Methods for Site Directed Mutagenesis in Eukaryotic Cells, Kmiec, U.S. Patent No. 5,565,350; *In Vivo* Homologous Sequence Targeting in Eukaryotic Cells; Zarling *et al.*, PCT/US93/03868. Likewise, a naturally occurring nucleic acid (e.g., a promoter) become isolated if it is introduced by non-naturally occurring means to a locus of the genome not native to that nucleic acid.

The term "protease inhibitor nucleic acids" means an isolated nucleic acid comprising a polynucleotide (a "protease inhibitor polynucleotide") encoding a polypeptide involved in protease inhibition.

As used herein, "localized within the chromosomal region defined by and including" with respect to particular markers includes reference to a contiguous length of a chromosome delimited by and including the stated markers.

As used herein, "marker" includes reference to a locus on a chromosome that serves to identify a unique position on the chromosome. A "polymorphic marker" includes reference to a marker which appears in multiple forms (alleles) such that different forms of the marker, when they are present in a homologous pair, allow transmission of each of the chromosomes in that pair to be followed. A genotype may be defined by use of a single or a plurality of markers.

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As used herein, "nucleic acid" includes reference to a deoxyribonucleotide or ribonucleotide polymer in either single- or double-stranded form, and unless otherwise limited, encompasses known analogues of natural nucleotides that hybridize to single-stranded nucleic acids in a manner similar to naturally occurring nucleotides (e.g., peptide
5 nucleic acids).

By "nucleic acid library" is meant a collection of isolated DNA or RNA molecules which comprise and substantially represent the entire transcribed fraction of a genome of a specified organism. Construction of exemplary nucleic acid libraries, such as genomic and cDNA libraries, is taught in standard molecular biology references such as Berger and
10 Kimmel, *Guide to Molecular Cloning Techniques, Methods in Enzymology*, Vol. 152, Academic Press, Inc., San Diego, CA (Berger); Sambrook *et al.*, *Molecular Cloning - A Laboratory Manual*, 2nd ed., Vol. 1-3 (1989); and *Current Protocols in Molecular Biology*, F.M. Ausubel *et al.*, Eds., Current Protocols, a joint venture between Greene Publishing Associates, Inc. and John Wiley & Sons, Inc. (1994 Supplement).

15 As used herein "operably linked" includes reference to a functional linkage between a promoter and a second sequence, wherein the promoter sequence initiates and mediates transcription of the DNA sequence corresponding to the second sequence. Generally, operably linked means that the nucleic acid sequences being linked are contiguous and, where necessary to join two protein coding regions, contiguous and in the
20 same reading frame.

As used herein, the term "plant" includes reference to whole plants, plant organs (e.g., leaves, stems, roots, etc.), seeds and plant cells and progeny of same. Plant cell, as used herein includes, without limitation, seeds suspension cultures, embryos, meristematic regions, callus tissue, leaves, roots, shoots,
25 gametophytes, sporophytes, pollen, and microspores. The class of plants which can be used in the methods of the invention is generally as broad as the class of higher plants amenable to transformation techniques, including both monocotyledonous and dicotyledonous plants. Particularly preferred is *Zea mays*.

As used herein, "polynucleotide" includes reference to a deoxyribopolynucleotide, ribopolynucleotide, or analogs thereof, that hybridize to nucleic acids in a manner similar
30 to naturally occurring nucleotides. A polynucleotide can be full-length or a sub-sequence of a native or heterologous structural or regulatory gene. Unless otherwise indicated, the

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term includes reference to the specified sequence as well as the complementary sequence thereof. Thus, DNAs or RNAs with backbones modified for stability or for other reasons are "polynucleotides" as that term is intended herein. Moreover, DNAs or RNAs comprising unusual bases, such as inosine, or modified bases, such as tritylated bases, to name just two examples, are polynucleotides as the term is used herein. It will be appreciated that a great variety of modifications have been made to DNA and RNA that serve many useful purposes known to those of skill in the art. The term polynucleotide as it is employed herein embraces such chemically, enzymatically or metabolically modified forms of polynucleotides, as well as the chemical forms of DNA and RNA characteristic of viruses and cells, including *inter alia*, simple and complex cells.

The terms "polypeptide", "peptide" and "protein" are used interchangeably herein to refer to a polymer of amino acid residues. The terms apply to amino acid polymers in which one or more amino acid residue is an artificial chemical analogue of a corresponding naturally occurring amino acid, as well as to naturally occurring amino acid polymers. Among the known modifications which may be present in polypeptides of the present are, to name an illustrative few, acetylation, acylation, ADP-ribosylation, amidation, covalent attachment of flavin, covalent attachment of a heme moiety, covalent attachment of a nucleotide or nucleotide derivative, covalent attachment of a lipid or lipid derivative, covalent attachment of phosphatidylinositol, cross-linking, cyclization, disulfide bond formation, demethylation, formation of covalent cross-links, formation of cystine, formation of pyroglutamate, formylation, gamma-carboxylation, glycosylation, GPI anchor formation, hydroxylation, iodination, methylation, myristoylation, oxidation, proteolytic processing, phosphorylation, prenylation, racemization, selenoylation, sulfation, transfer-RNA mediated addition of amino acids to proteins such as arginylation, and ubiquitination. Such modifications are well known to those of skill and have been described in great detail in the scientific literature. Several particularly common modifications, glycosylation, lipid attachment, sulfation, gamma-carboxylation of glutamic acid residues, hydroxylation and ADP-ribosylation, for instance, are described in most basic texts, such as, for instance *Proteins - Structure and Molecular Properties*, 2nd ed., T. E. Creighton, W. H. Freeman and Company, New York (1993). Many detailed reviews are available on this subject, such as, for example, those provided by Wold, F., *Posttranslational Protein Modifications: Perspectives and Prospects*, pp. 1-12 in *Posttranslational Covalent Modification of Proteins*,

11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30

B. C. Johnson, Ed., Academic Press, New York (1983); Seifter *et al.*, Meth. Enzymol. 182: 626-646 (1990) and Rattan *et al.*, *Protein Synthesis: Posttranslational Modifications and Aging*, Ann. N.Y. Acad. Sci. 663: 48-62 (1992). It will be appreciated, as is well known and as noted above, that polypeptides are not always entirely linear. For instance, polypeptides
5 may be branched as a result of ubiquitination, and they may be circular, with or without branching, generally as a result of posttranslation events, including natural processing event and events brought about by human manipulation which do not occur naturally. Circular, branched and branched circular polypeptides may be synthesized by non-translation natural process and by entirely synthetic methods, as well. Modifications can occur anywhere in a
10 polypeptide, including the peptide backbone, the amino acid side-chains and the amino or carboxyl termini. In fact, blockage of the amino or carboxyl group in a polypeptide, or both, by a covalent modification, is common in naturally occurring and synthetic polypeptides and such modifications may be present in polypeptides of the present invention, as well. For instance, the amino terminal residue of polypeptides made in *E. coli* or other cells, prior
15 to proteolytic processing, almost invariably will be N-formylmethionine. During post-translational modification of the peptide, a methionine residue at the NH₂-terminus may be deleted. Accordingly, this invention contemplates the use of both the methionine-containing and the methionineless amino terminal variants of the protein of the invention. In general, as used herein, the term polypeptide encompasses all such modifications,
20 particularly those that are present in polypeptides synthesized by expressing a polynucleotide in a host cell.

As used herein "promoter" includes reference to a region of DNA upstream from the start of transcription and involved in recognition and binding of RNA polymerase and other proteins to initiate transcription. A "plant promoter" is a promoter capable of
25 initiating transcription in plant cells. Examples of promoters under developmental control include promoters that preferentially initiate transcription in certain tissues, such as leaves, roots, seeds, fibers, xylem vessels, tracheids, or sclerenchyma. Such promoters are referred to as "tissue preferred". Promoters which initiate transcription only in certain tissue are referred to as "tissue specific". A "cell type" specific promoter is primarily
30 drives expression in certain cell types in one or more organs, for example, vascular cells in roots or leaves. An "inducible" promoter is a promoter which is under environmental control. Examples of environmental conditions that may effect transcription by inducible

promoters include anaerobic conditions or the presence of light. Tissue specific, cell type specific, and inducible promoters constitute the class of "non-constitutive" promoters. A "constitutive" promoter is a promoter which is active under most environmental conditions.

5 The terms "polypeptide involved in protease inhibition" or "protease inhibitor polypeptide" refer to one or more proteins, in glycosylated or non-glycosylated form, acting as a protease inhibitor. Examples are included as, but not limited to: chymotrypsin inhibitor, trypsin inhibitor, protease inhibitor, pre-pro-proteinase inhibitor I, subtilisin-chymotrypsin inhibitor, tumor-related protein, genetic tumor-related proteinase inhibitor, 10 subtilisin inhibitor, endopeptidase inhibitor, serine protease inhibitor, wound-inducible proteinase inhibitor, and eglin c. The term is also inclusive of fragments, variants, homologs, alleles or precursors (e.g., preproteins or proproteins) thereof. A "protease inhibitor protein" comprises a protease inhibitor polypeptide.

As used herein "recombinant" includes reference to a cell, or nucleic acid, or 15 vector, that has been modified by the introduction of a heterologous nucleic acid or the alteration or placement of a native nucleic acid to a form or to a locus not native to that cell, or that the cell is derived from a cell so modified. Thus, for example, recombinant cells express genes that are not found in identical form within the native (non-recombinant) form of the cell or express native genes that are otherwise abnormally 20 expressed, under expressed or not expressed at all. The term "recombinant" as used herein does not encompass the alteration of the cell, nucleic acid or vector by naturally occurring events (e.g., spontaneous mutation, natural transformation/transduction/transposition) such as those occurring without direct human intervention.

25 As used herein, a "recombinant expression cassette" is a nucleic acid construct, generated recombinantly or synthetically, with a series of specified nucleic acid elements which permit transcription of a particular nucleic acid in a target cell. The recombinant expression cassette can be incorporated into a plasmid, chromosome, mitochondrial DNA, plastid DNA, virus, or nucleic acid fragment. Typically, the recombinant expression 30 cassette portion of the expression vector includes, among other sequences, a nucleic acid to be transcribed, and a promoter.

The term "residue" or "amino acid residue" or "amino acid" are used interchangeably herein to refer to an amino acid that is incorporated into a protein, polypeptide, or peptide (collectively "protein"). The amino acid may be a naturally occurring amino acid and, unless otherwise limited, may encompass known analogs of natural amino acids that can function in a similar manner as naturally occurring amino acids.

The term "selectively hybridizes" includes reference to hybridization, under stringent hybridization conditions, of a nucleic acid sequence to a specified nucleic acid target sequence to a detectably greater degree (e.g., at least 2-fold over background) than its hybridization to non-target nucleic acid sequences and to the substantial exclusion of non-target nucleic acids. Selectively hybridizing sequences typically have about at least 80% sequence identity, preferably 90% sequence identity, and most preferably 100% sequence identity (i.e., complementary) with each other.

The terms "stringent conditions" or "stringent hybridization conditions" includes reference to conditions under which a probe will hybridize to its target sequence, to a detectably greater degree than other sequences (e.g., at least 2-fold over background). Stringent conditions are sequence-dependent and will be different in different circumstances. Longer sequences hybridize specifically at higher temperatures. Generally, stringent conditions are selected to be about 5 °C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength and pH. The T_m is the temperature (under defined ionic strength and pH) at which 50% of a complementary target sequence hybridizes to a perfectly matched probe. Typically, stringent conditions will be those in which the salt concentration is less than about 1.0 M Na ion, typically about 0.01 to 1.0 M Na ion concentration (or other salts) at pH 7.0 to 8.3 and the temperature is at least about 30°C for short probes (e.g., 10 to 50 nucleotides) and at least about 60°C for long probes (e.g., greater than 50 nucleotides). Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide. Exemplary low stringency conditions include hybridization with a buffer solution of 30% formamide, 1 M NaCl, 1% SDS at 37°C, and a wash in 2X SSC at 50°C. Exemplary high stringency conditions include hybridization in 50% formamide, 1 M NaCl, 1% SDS at 37°C, and a wash in 0.1X SSC at 60°C.

Stringent hybridization conditions in the context of nucleic acid hybridization assay formats are sequence dependent, and are different under different environmental parameters. Longer sequences hybridize selectively at higher temperatures. An extensive guide to the hybridization of nucleic acids is found in Tijssen, *Laboratory Techniques in*
5 *Biochemistry and Molecular Biology--Hybridization with Nucleic Acid Probes*, Part I, Chapter 2 "Overview of principles of hybridization and the strategy of nucleic acid probe assays", Elsevier, New York (1993).

The terms "transfection" or "transformation" include reference to the introduction of a nucleic acid into a eukaryotic or prokaryotic cell where the nucleic acid may be
10 incorporated into the genome of the cell (e.g., chromosome, plasmid, plastid or mitochondrial DNA), converted into an autonomous replicon, or transiently expressed (e.g., transfected mRNA).

As used herein, "transgenic plant" includes reference to a plant which comprises within its genome a heterologous polynucleotide. Generally, the heterologous
15 polynucleotide is stably integrated within the genome such that the polynucleotide is passed on to successive generations. The heterologous polynucleotide may be integrated into the genome alone or as part of a recombinant expression cassette. "Transgenic" is used herein to include any cell, cell line, callus, tissue, plant part or plant, the genotype of which has been altered by the presence of heterologous nucleic acid including those
20 transgenics initially so altered as well as those created by sexual crosses or asexual propagation from the initial transgenic. The term "transgenic" as used herein does not encompass the alteration of the genome (chromosomal or extra-chromosomal) by conventional plant breeding methods or by naturally occurring events such as random cross-fertilization,
25 non-recombinant viral infection, non-recombinant bacterial transformation, non-recombinant transposition, or spontaneous mutation.

As used herein, "vector" includes reference to a nucleic acid used in transfection of a host cell and into which can be inserted a polynucleotide. Vectors are often replicons. Expression vectors permit transcription of a nucleic acid inserted therein.

30 The following terms are used to describe the sequence relationships between two or more nucleic acids or polynucleotides: (a) "reference sequence", (b) "comparison

window”, (c) “sequence identity”, (d) “percentage of sequence identity”, and (e) “substantial identity”.

(a) As used herein, “reference sequence” is a defined sequence used as a basis for sequence comparison. A reference sequence may be a subset or the entirety of a specified sequence; for example, as a segment of a full-length cDNA or gene sequence, or the complete cDNA or gene sequence.

(b) As used herein, “comparison window” means includes reference to a contiguous and specified segment of a polynucleotide sequence, wherein the polynucleotide sequence may be compared to a reference sequence and wherein the portion of the polynucleotide sequence in the comparison window may comprise additions or deletions (i.e., gaps) compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. Generally, the comparison window is at least 20 contiguous nucleotides in length, and optionally can be 30, 40, 50, 100, or longer. Those of skill in the art understand that to avoid a high similarity to a reference sequence due to inclusion of gaps in the polynucleotide sequence a gap penalty is typically introduced and is subtracted from the number of matches.

Methods of alignment of sequences for comparison are well-known in the art. Optimal alignment of sequences for comparison may be conducted by the local homology algorithm of Smith and Waterman, Adv. Appl. Math. 2: 482 (1981); by the homology alignment algorithm of Needleman and Wunsch, J. Mol. Biol. 48: 443 (1970); by the search for similarity method of Pearson and Lipman, Proc. Natl. Acad. Sci. 85: 2444 (1988); by computerized implementations of these algorithms, including, but not limited to: CLUSTAL in the PC/Gene program by Intelligenetics, Mountain View, California, GAP, BESTFIT, BLAST, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group (GCG), 575 Science Dr., Madison, Wisconsin, USA; the CLUSTAL program is well described by Higgins and Sharp, Gene 73: 237-244 (1988); Higgins and Sharp, CABIOS 5: 151-153 (1989); Corpet, *et al.*, Nucleic Acids Research 16: 10881-90 (1988); Huang, *et al.*, Computer Applications in the Biosciences 8: 155-65 (1992), and Pearson, *et al.*, Methods in Molecular Biology 24: 307-331 (1994); preferred computer alignment methods also include the BLASTP, BLASTN, and BLASTX algorithms. Altschul, *et al.*, J. Mol. Biol. 215: 403-410 (1990). Alignment is also often performed by inspection and manual alignment.

(c) As used herein, "sequence identity" or "identity" in the context of two nucleic acid or polypeptide sequences includes reference to the residues in the two sequences which are the same when aligned for maximum correspondence over a specified comparison window. When percentage of sequence identity is used in reference to proteins it is recognized that residue positions which are not identical often differ by conservative amino acid substitutions, where amino acid residues are substituted for other amino acid residues with similar chemical properties (e.g. charge or hydrophobicity) and therefore do not change the functional properties of the molecule. Where sequences differ in conservative substitutions, the percent sequence identity may be adjusted upwards to correct for the conservative nature of the substitution. Sequences which differ by such conservative substitutions are said to have "sequence similarity" or "similarity". Means for making this adjustment are well-known to those of skill in the art. Typically this involves scoring a conservative substitution as a partial rather than a full mismatch, thereby increasing the percentage sequence identity. Thus, for example, where an identical amino acid is given a score of 1 and a non-conservative substitution is given a score of zero, a conservative substitution is given a score between zero and 1. The scoring of conservative substitutions is calculated, e.g., according to the algorithm of Meyers and Miller, Computer Applic. Biol. Sci., 4: 11-17 (1988) e.g. as implemented in the program PC/GENE (Intelligenetics, Mountain View, California, USA).

(d) As used herein, "percentage of sequence identity" means the value determined by comparing two optimally aligned sequences over a comparison window, wherein the portion of the polynucleotide sequence in the comparison window may comprise additions or deletions (i.e., gaps) as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical nucleic acid base or amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison and multiplying the result by 100 to yield the percentage of sequence identity.

(e) (i) The term "substantial identity" of polynucleotide sequences means that a polynucleotide comprises a sequence that has at least 70% sequence identity, preferably at least 80%, more preferably at least 90% and most preferably at least 95%,

compared to a reference sequence using one of the alignment programs described using standard parameters. One of skill will recognize that these values can be appropriately adjusted to determine corresponding identity of proteins encoded by two nucleotide sequences by taking into account codon degeneracy, amino acid similarity, reading frame positioning and the like. Substantial identity of amino acid sequences for these purposes normally means sequence identity of at least 60%, more preferably at least 70%, 80%, 90%, and most preferably at least 95%. Polypeptides which are "substantially similar" share sequences as noted above except that residue positions which are not identical may differ by conservative amino acid changes.

Another indication that nucleotide sequences are substantially identical is if two molecules hybridize to each other under stringent conditions. Generally, stringent conditions are selected to be about 5°C to about 20°C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength and pH. The T_m is the temperature (under defined ionic strength and pH) at which 50% of the target sequence hybridizes to a perfectly matched probe. Typically, stringent wash conditions are those in which the salt concentration is about 0.02 molar at pH 7 and the temperature is at least about 50, 55, or 60°C. However, nucleic acids which do not hybridize to each other under stringent conditions are still substantially identical if the polypeptides which they encode are substantially identical. This may occur, *e.g.*, when a copy of a nucleic acid is created using the maximum codon degeneracy permitted by the genetic code. One indication that two nucleic acid sequences are substantially identical is that the polypeptide which the first nucleic acid encodes is immunologically cross reactive with the polypeptide encoded by the second nucleic acid.

(e) (ii) The terms "substantial identity" in the context of a peptide indicates that a peptide comprises a sequence with at least 70% sequence identity to a reference sequence, preferably 80%, more preferably 85%, most preferably at least 90% or 95% sequence identity to the reference sequence over a specified comparison window. Preferably, optimal alignment is conducted using the homology alignment algorithm of Needleman and Wunsch, J. Mol. Biol. 48: 443 (1970). An indication that two peptide sequences are substantially identical is that one peptide is immunologically reactive with antibodies raised against the second peptide. Thus, a peptide is substantially identical to a

second peptide, for example, where the two peptides differ only by a conservative substitution.

5 It has been unexpectedly discovered that a protease inhibitor can be modified to enhance its content of essential amino acids coupled with reduction in protease inhibitor activity. In a preferred embodiment of the present invention, derivatives of the protease inhibitor, CI-2, simultaneously exhibit both enhanced essential amino acid content as well as decreased protease inhibitor activity. The present compounds are thus excellent
10 candidates for enhancing the nutritional value of feed.

 The present invention provides, *inter alia*, compositions and methods for modulating (i.e., increasing or decreasing) the total levels of essential amino acids and/or altering the ratios of essential amino acids in plants. Thus, the present invention provides utility in such exemplary applications as improving the nutritional properties of fodder
15 crops, increasing the value of plant material for pulp and paper production, altering the protease inhibitory activity, as well as for improving the utility of plant material where the amount of essential amino acids or composition is important, such as the use of plant as a feed. In particular, protease inhibitor polypeptides may be expressed at times or in quantities which are not characteristic of natural plants.

20 The present invention also provides isolated nucleic acid comprising polynucleotides of sufficient length and complementarity to a protease inhibitor gene, to use as probes or amplification primers in the detection, quantitation, or isolation of gene transcripts. For example, isolated nucleic acids of the present invention can be used as probes in detecting deficiencies in the level of mRNA in screenings for desired transgenic
25 plants, for detecting mutations in the gene (e.g., substitutions, deletions, or additions), for monitoring upregulation of protease inhibition in screening assays for compounds affecting protease inhibition, or for use as molecular markers in plant breeding programs. The isolated nucleic acids of the present invention can also be used for recombinant expression of protease inhibitor polypeptides for use as immunogens in the preparation
30 and/or screening of antibodies. The isolated nucleic acids of the present invention can also be employed for use in sense or antisense suppression of one or more protease inhibitor genes in a host cell, tissue, or plant. Further, using a primer specific to an

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30

insertion sequence (e.g., transposon) and a primer which specifically hybridizes to an isolated nucleic acid of the present invention, one can use nucleic acid amplification to identify insertion sequence inactivated protease inhibitor genes from a cDNA library prepared from insertion sequence mutagenized plants. Progeny seed from the plants comprising the desired inactivated gene can be grown to a plant to study the phenotypic changes characteristic of that inactivation. See, *Tools to Determine the Function of Genes*, 1995 Proceedings of the Fiftieth Annual Corn and Sorghum Industry Research Conference, American Seed Trade Association, Washington, D.C., 1995.

The present invention also provides isolated proteins comprising polypeptides having a minimal amino acid sequence from the polypeptides involved in protease inhibition as disclosed herein. The present invention also provides proteins comprising at least one epitope from a polypeptide involved in protease inhibition. The proteins of the present invention can be employed in assays for enzyme agonists or antagonists of enzyme function, or for use as immunogens or antigens to obtain antibodies specifically immunoreactive with a protein of the present invention. Such antibodies can be used in assays for expression levels, for identifying and/or isolating nucleic acids of the present invention from expression libraries, or for purification of polypeptides involved in protease inhibition. In a preferred embodiment of the present invention, the present protein has both elevated essential amino acid content and reduced protease inhibitor activity.

The isolated nucleic acids of the present invention can be used over a broad range of plant types, including species from the genera *Cucurbita*, *Rosa*, *Vitis*, *Juglans*, *Fragaria*, *Lotus*, *Medicago*, *Onobrychis*, *Trifolium*, *Trigonella*, *Vigna*, *Citrus*, *Linum*, *Geranium*, *Manihot*, *Daucus*, *Arabidopsis*, *Brassica*, *Raphanus*, *Sinapis*, *Atropa*, *Capsicum*, *Datura*, *Hyoscyamus*, *Lycopersicon*, *Nicotiana*, *Solanum*, *Petunia*, *Digitalis*, *Majorana*, *Ciahorium*, *Helianthus*, *Lactuca*, *Bromus*, *Asparagus*, *Antirrhinum*, *Heterocallis*, *Nemesis*, *Pelargonium*, *Panieum*, *Pennisetum*, *Ranunculus*, *Senecio*, *Salpiglossis*, *Cucumis*, *Browaalia*, *Glycine*, *Pisum*, *Phaseolus*, *Lolium*, *Oryza*, *Zea*, *Avena*, *Hordeum*, *Secale*, *Triticum*, *Sorghum*, *Picea*, and *Populus*.

30

The isolated nucleic acids of the present invention can be used over a broad range of polypeptide types, including anti microbial peptides such as those described and incorporated by reference in Rao, G., Antimicrobial Peptides; Molecular Plant-Microbe Interactions 8: 6-13 (1995).

5

Protease Inhibitor Nucleic Acids

The present invention provides, *inter alia*, isolated and/or heterologous nucleic acids of RNA, DNA, and analogs and/or chimeras thereof, comprising a protease inhibitor polynucleotide encoding such proteins as: chymotrypsin inhibitor, trypsin inhibitor, protease inhibitor, pre-pro-proteinase inhibitor I, subtilisin-chymotrypsin inhibitor, tumor-related protein, genetic tumor-related proteinase inhibitor, subtilisin inhibitor, endopeptidase inhibitor, serine protease inhibitor, wound-inducible proteinase inhibitor, and eglin c. The protease inhibitor nucleic acids of the present invention comprise protease inhibitor polynucleotides which, are inclusive of:

(a) a polynucleotide encoding a protease inhibitor polypeptide of SEQ ID NOS: 2,4,6,8,10, or 12,16,18,20,22,24 and conservatively modified and polymorphic variants thereof, including exemplary polynucleotides of SEQ ID NOS: 1,3,5,7,9 and 11, 15,17,19,21, 23 and conservative changes

(b) a polynucleotide which is the product of amplification from a *Zea mays* nucleic acid library using primer pairs from amongst the consecutive pairs from SEQ ID NOS: 25 and 26, which amplify polynucleotides having substantial identity to polynucleotides from amongst those having SEQ ID NOS: 1,3,5,7,9 or 11,15,17,19,21, 23

(c) a polynucleotide which selectively hybridizes under stringent hybridization conditions consisting of washing in a salt concentration of about 0.02 molar at pH 7 at 50°C, to a polynucleotide of (a) or (b);

(d) a polynucleotide having at least 60% sequence identity with Sequence ID Nos. 1, 3, 5, 7, 9, 11, 15, 17, 19, 21 or 23;

(e) a polynucleotide encoding a protein having a specified number of contiguous amino acids from a prototype polypeptide, wherein the protein is specifically recognized by antisera elicited by presentation of the protein and wherein the protein does

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30

not detectably immunoreact to antisera which has been fully immunosorbed with the protein;

(f) complementary sequences of polynucleotides of (a), (b), (c), (d), or (e);

and

5 (g) a polynucleotide comprising at least 20 contiguous nucleotides from a polynucleotide of Sequence ID Nos. 1, 3, 5, 7, 9, 11, 15, 17, 19, 21 or 23.

A. Polynucleotides Encoding A Protease inhibitor Protein of SEQ ID NOS: 2,4,6,8,10 and 12,16,18,20,22,24 or Conservatively Modified or Polymorphic Variants Thereof

10 As indicated in (a), *supra*, the present invention provides isolated and/or heterologous nucleic acids comprising protease inhibitor polynucleotides, wherein the polynucleotides encode the protease inhibitor polypeptides disclosed herein as SEQ ID NOS: 2,4,6,8,10 and 12,16,18,20,22,24 or conservatively modified or polymorphic variants thereof. Those of skill in the art will recognize that the degeneracy of the genetic
15 code allows for a plurality of polynucleotides to encode for the identical amino acid sequence. Thus, the present invention includes protease inhibitor polynucleotides of SEQ ID NOS: 1,3,5,7,9 and 11, 15,17,19,21, 23 and silent variations of polynucleotides encoding a protease inhibitor polypeptide of SEQ ID NOS: 2,4,6,8,10 and
20 12,16,18,20,22,24. The present invention further provides isolated and/or heterologous nucleic acids comprising protease inhibitor polynucleotides encoding conservatively modified variants of a protease inhibitor polypeptide of SEQ ID NOS: 2,4,6,8,10 and 12, 16,18,20,22,24. Additionally, the present invention further provides isolated and/or
heterologous nucleic acids comprising protease inhibitor polynucleotides encoding one or more polymorphic (allelic) variants of protease inhibitor polypeptides/polynucleotides.

25

B. Polynucleotides Amplified from a Zea mays Nucleic Acid Library

As indicated in (b), *supra*, the present invention provides isolated and/or heterologous nucleic acids comprising protease inhibitor polynucleotides, wherein the polynucleotides are amplified from a *Zea mays* nucleic acid library. The nucleic acid
30 library may be a cDNA library, a genomic library, or a library generally constructed from nuclear transcripts at any stage of intron processing. Nucleic acid libraries from other plants, both monocots and dicots could also be used in a similar fashion. The

polynucleotides of the present invention include those amplified using the following primer pairs:

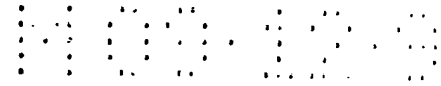
SEQ ID NOS: 25 and 26 which yield an amplicon comprising a sequence having substantial identity to SEQ ID NOS: 7,9, and 11.

5 Thus, the present invention provides protease inhibitor synthetic polynucleotides having the sequence of the gene, a nuclear transcript, a cDNA, or complementary sequences thereof. In preferred embodiments, the nucleic acid library is constructed from *Zea mays*, such as lines B73, PHRE1, A632, BMS-P2#10, and W23, each of which are known and publicly available. In particularly preferred embodiments, the library is
10 constructed from tissue such as root, leaf, or tassel, or embryonic tissue.

The amplification products can be translated using expression systems well known to those of skill in the art and as discussed, *infra*. The resulting translation products can be confirmed as protease inhibitor polypeptides of the present invention by, for example, assaying for the appropriate inhibition activity or verifying the presence of a linear
15 epitope which is specific to a protease inhibitor polypeptide using standard immunoassay methods.

Those of ordinary skill will appreciate that primers which selectively amplify, under stringent conditions, the polynucleotides of the present invention (and their complements) can be constructed by reference to the sequences provided herein at SEQ
20 ID NOS: 1,3,5,7,9 and 11. In preferred embodiments, the primers will be constructed to anneal with the first three contiguous nucleotides at their 5' terminal end's to the first codon encoding the carboxy or amino terminal amino acid residue (or the complements thereof) of the polynucleotides of the present invention. Typically, such primers are at least 15 nucleotides in length. The primer length in nucleotides is selected from the group
25 of integers consisting of from at least 15 to 90. Thus, the primers can be at least 15, 18, 20, 25, 30, 40, 50, 60, 70, 80, or 90 nucleotides in length.

The amplification primers may optionally be elongated in the 3' direction with contiguous nucleotide sequences from polynucleotide sequences of SEQ ID NOS: 1,3,5,7,9 and 11, 15,17,19,21, from which they are derived. The number of nucleotides
30 by which the primers can be elongated is selected from the group of integers consisting of from at least 1 to 25. Thus, for example, the primers can be elongated with an additional 1, 5, 10, or 15 nucleotides. Those of skill will recognize that a lengthened primer



sequence can be employed to increase specificity of binding (i.e., annealing) to a target sequence.

C. Polynucleotides Which Selectively Hybridize to a Polynucleotide of (A) or (B)

5 As indicated in (c), *supra*, the present invention provides isolated and/or heterologous nucleic acids comprising protease inhibitor polynucleotides, wherein the polynucleotides selectively hybridize, under selective hybridization conditions, to a protease inhibitor polynucleotide of paragraphs (A) or (B) as discussed, *supra*. Thus, the polynucleotides of this embodiment can be used for isolating, detecting, and/or
10 quantifying nucleic acids comprising the polynucleotides of (A) or (B). Low stringency hybridization conditions are typically, but not exclusively, employed with sequences having relatively small sequence identity. Moderate and high stringency conditions can optionally be employed for sequences of greater identity. Low stringency conditions allow selective hybridization of sequences having about 70% sequence identity.

15

D. Polynucleotides Having at Least 60% Sequence Identity with the Polynucleotides of (A), (B) or (C)

 As indicated in (d), *supra*, the present invention provides isolated and/or heterologous nucleic acids comprising protease inhibitor polynucleotides, wherein the
20 polynucleotides have a specified identity at the nucleotide level to a polynucleotide as disclosed above in paragraphs (A), (B), (C), or (D). The percentage of identity to a reference sequence is at least 60% and, rounded upwards to the nearest integer, can be expressed as an integer selected from the group of integers consisting of from 60 to 99. Thus, for example, the percentage of identity to a reference sequence can be at least 70%,
25 75%, 80%, 85%, 90%, or 95%.

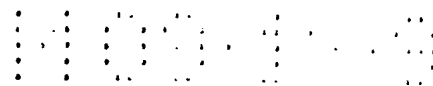
 The protease inhibitor polynucleotide optionally encodes a protein having a molecular weight as the unglycosylated protein within 20% of the molecular weight of the truncated or full-length protease inhibitor polypeptides as disclosed herein (e.g., SEQ ID
30 NOS: 2,4,6,8,10 and 12). Preferably, the molecular weight is within 15% of a full length protease inhibitor polypeptide, more preferably within 10% or 5%, and most preferably

Optionally, the protease inhibitor polynucleotides of this embodiment will encode a protein having an inhibitory activity less than or equal to 20%, 30%, 40%, or 50% of the native, endogenous (i.e., non-isolated), full-length protease inhibitor polypeptide. Determination of protein inhibition can be determined by any number of means well known to those of skill in the art.

As indicated in (f), *supra*, the present invention provides isolated and/or heterologous nucleic acids comprising protease inhibitor polynucleotides, wherein the polynucleotides are complementary to the polynucleotides of paragraphs A-E, above. As those of skill in the art will recognize, complementary sequences base-pair throughout the entirety of their length with the polynucleotides of (A)-(E) (i.e., have 100% sequence identity). Complementary bases associate through hydrogen bonding in double stranded nucleic acids. For example, the following base pairs are complementary: guanine and cytosine; adenine and thymine; and adenine and uracil.

As indicated in (h), *supra*, the present invention provides isolated and/or heterologous nucleic acids comprising protease inhibitor polynucleotides, wherein the polynucleotide comprises at least 15 contiguous bases from the polynucleotides of (A) through (F) as discussed above. The length of the polynucleotide is given as an integer selected from the group consisting of from at least 15 to the length of the nucleic acid sequence from which the protease inhibitor polynucleotide is a subsequence of. Thus, for example, polynucleotides of the present invention are inclusive of polynucleotides comprising at least 15, 20, 25, 30, 40, 50, 60, 75, or 100 contiguous nucleotides in length from the polynucleotides of (A)-(F). Optionally, the number of such subsequences encoded by a polynucleotide of the instant embodiment can be any integer selected from the group consisting of from 1 to 20, such as 2, 3, 4, or 5.

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The isolated and/or heterologous protease inhibitor nucleic acids of the present invention can be made using (a) standard recombinant methods, (b) synthetic techniques, or combinations thereof. In some embodiments, the protease inhibitor polynucleotides of the present invention will be cloned, amplified, or otherwise constructed from a plant.

- 5 The preferred plants are barley and *Zea mays*, such as inbred line B73 which is publicly known and available. Particularly preferred is the use of *Zea mays* tissue such as roots, leaves, tassels, seeds or embryonic tissue.

A. Recombinant Methods for Constructing Protease inhibitor Nucleic Acids

- 10 The isolated and/or heterologous nucleic acid compositions of this invention, such as RNA, cDNA, genomic DNA, or a hybrid thereof, can be obtained from plant biological sources using any number of cloning methodologies known to those of skill in the art.

- The isolation of protease inhibitor polynucleotides may be accomplished by a number of techniques. For instance, oligonucleotide probes based on the sequences
15 disclosed here can be used to identify the desired gene in a cDNA or genomic DNA library. To construct genomic libraries, large segments of genomic DNA are generated by random fragmentation, e.g. using restriction endonucleases, and are ligated with vector DNA to form concatemers that can be packaged into the appropriate vector. To prepare a
20 cDNA library, mRNA is isolated from the desired organ, such as sclerenchyma and a cDNA library which contains the gene encoding for a protease inhibitor protein (i.e., the protease inhibitor gene) is prepared from the mRNA. Alternatively, cDNA may be prepared from mRNA extracted from other tissues in which protease inhibitor genes or homologs are expressed.

- The DNA or genomic library can then be screened using a probe based upon the
25 sequence of a cloned protease inhibitor polynucleotide such as those disclosed herein. Probes may be used to hybridize with genomic DNA or cDNA sequences to isolate homologous genes in the same or different plant species. Those of skill in the art will appreciate that various degrees of stringency of hybridization can be employed in the assay; and either the hybridization or the wash medium can be stringent. As the
30 conditions for hybridization become more stringent, there must be a greater degree of complementarity between the probe and the target for duplex formation to occur. The degree of stringency can be controlled by temperature, ionic strength, pH and the presence

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of a partially denaturing solvent such as formamide. For example, the stringency of hybridization is conveniently varied by changing the polarity of the reactant solution through manipulation of the concentration of formamide within the range of 0% to 50%.

Cloning methodologies to accomplish these ends, and sequencing methods to
5 verify the sequence of nucleic acids are well known in the art. Examples of appropriate cloning and sequencing techniques, and instructions sufficient to direct persons of skill through many cloning exercises are found in Sambrook, *et al.*, *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory Vols. 1-3 (1989), Methods in Enzymology, Vol. 152: *Guide to Molecular Cloning Techniques*, Berger and Kimmel,
10 Eds., San Diego: Academic Press, Inc. (1987), *Current Protocols in Molecular Biology*, Ausubel, *et al.*, Eds., Greene Publishing and Wiley-Interscience, New York (1987); *Plant Molecular Biology: A Laboratory Manual*, Clark, Ed., Springer-Verlag, Berlin (1997).

The nucleic acids of interest can also be amplified from nucleic acid samples using amplification techniques. For instance, polymerase chain reaction (PCR)
15 technology can be used to amplify the sequences of protease inhibitor polynucleotides of the present invention and related genes directly from genomic DNA or cDNA libraries. PCR and other *in vitro* amplification methods may also be useful, for example, to clone nucleic acid sequences that code for proteins to be expressed, to make nucleic acids to use as probes for detecting the presence of the desired mRNA in samples, for nucleic acid
20 sequencing, or for other purposes.

The degree of complementarity (sequence identity) required for detectable binding will vary in accordance with the stringency of the hybridization medium and/or wash medium. The degree of complementarity will optimally be 100 percent; however, it should be understood that minor sequence variations in the probes and primers may be
25 compensated for by reducing the stringency of the hybridization and/or wash medium.

Examples of techniques sufficient to direct persons of skill through *in vitro* amplification methods are found in Berger, Sambrook, and Ausubel, as well as Mullis *et al.*, U.S. Patent No. 4,683,202 (1987); *PCR Protocols A Guide to Methods and Applications*, Innis *et al.*, Eds., Academic Press Inc., San Diego, CA (1990); Arnheim &
30 Levinson, *C&EN* pp. 36-47 (October 1, 1990).

B. Synthetic Methods for Constructing Protease inhibitor Nucleic Acids

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50

The isolated nucleic acids of the present invention can also be prepared by direct chemical synthesis by methods such as the phosphotriester method of Narang *et al.*, Meth. Enzymol. 68: 90-99 (1979) and the phosphodiester method of Brown *et al.*, Meth. Enzymol. 68: 109-151 (1979). The isolated nucleic acids of the present invention

5 can also be modified through methods such as site directed mutagenesis, error prone PCR and known to one of skill.

Recombinant Expression Cassettes

10 The present invention further provides recombinant expression cassettes comprising a protease inhibitor nucleic acid of the present invention. A nucleic acid sequence coding for the desired protease inhibitor polynucleotide, for example a cDNA or a genomic sequence encoding a full length protease inhibitor protein, can be used to construct a recombinant expression cassette which can be introduced into the desired host

15 cell. A recombinant expression cassette will typically comprise a protease inhibitor polynucleotide operably linked to transcriptional initiation regulatory sequences which will direct the transcription of the protease inhibitor polynucleotide in the intended host cell, such as tissues of a transformed plant.

For example, plant expression vectors may include (1) a cloned plant gene

20 under the transcriptional control of 5' and 3' regulatory sequences and (2) a dominant selectable marker. Such plant expression vectors may also contain, if desired, a promoter regulatory region (e.g., one conferring inducible or constitutive, environmentally- or developmentally-regulated, or cell- or tissue-specific/selective expression), a transcription initiation start site, a ribosome

25 binding site, an RNA processing signal, a transcription termination site, and/or a polyadenylation signal. Highly preferred plant expression cassettes will be designed to include one or more selectable marker genes, such as kanamycin resistance or herbicide tolerance genes.

30 A plant promoter fragment may be employed which will direct expression of the protease inhibitor polynucleotide in all tissues of a regenerated plant. Such promoters are referred to herein as "constitutive" promoters and are active under most environmental

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conditions and states of development or cell differentiation. Examples of constitutive promoters include the cauliflower mosaic virus (CaMV) 35S transcription initiation region, the 1'- or 2'- promoter derived from T-DNA of *Agrobacterium tumefaciens*, the ubiquitin 1 promoter, the Smas promoter, the cinnamyl alcohol dehydrogenase promoter
5 (U.S. Patent No. 5,683,439), the *Nos* promoter, the pEmu promoter, the rubisco promoter, the GRP1-8 promoter, and other transcription initiation regions from various plant genes known to those of skill. In a preferred embodiment, the gamma zein promoter of maize would be used.

Alternatively, the plant promoter may direct expression of the protease inhibitor
10 polynucleotide in a specific tissue or may be otherwise under more precise environmental or developmental control. Examples of promoters under developmental control include promoters that initiate transcription only, or preferentially, in certain tissues, such as leaves, roots, fruit, seeds, or flowers. The operation of a promoter may also vary depending on its location in the genome. Thus, an inducible promoter may become fully
15 or partially constitutive in certain locations.

Both heterologous and non-heterologous (i.e., endogenous) promoters can be employed to direct expression of the protease inhibitor nucleic acids of the present invention. These promoters can also be used, for example, in recombinant expression cassettes to drive expression of antisense nucleic acids to reduce, increase, or alter
20 protease inhibitor content and/or composition in a desired tissue

Methods for identifying promoters with a particular expression pattern, in terms of, e.g., tissue type, cell type, stage of development, and/or environmental conditions, are well known in the art. See, e.g., *The Maize Handbook*, Chapters 114-115, Freeling and Walbot, Eds., Springer, New York (1994); *Corn and Corn Improvement*, 3rd
25 edition, Chapter 6, Sprague and Dudley, Eds., American Society of Agronomy, Madison, Wisconsin (1988). A typical step in promoter isolation methods is identification of gene products that are expressed with some degree of specificity in the target tissue. Amongst the range of methodologies are: differential hybridization to cDNA libraries; subtractive hybridization; differential display; differential 2-D gel electrophoresis; DNA probe arrays;
30 and isolation of proteins known to be expressed with some specificity in the target tissue. Such methods are well known to those of skill in the art. Commercially available

products for identifying promoters are known in the art such as CloneTech's (Palo Alto, CA) PROMOTERFINDER DNA Walking Kit.

Once promoter and/or gene sequences are known, a region of suitable size is
5 selected from the genomic DNA that is 5' to the transcriptional start, or the translational
start site, and such sequences are then linked to a coding sequence. If the transcriptional
start site is used as the point of fusion, any of a number of possible 5' untranslated regions
can be used in between the transcriptional start site and the partial coding sequence. If the
translational start site at the 3' end of the specific promoter is used, then it is linked
10 directly to the methionine start codon of a coding sequence.

If polypeptide expression is desired, it is generally desirable to include a
polyadenylation region at the 3'-end of the protease inhibitor polynucleotide coding
region. An intron sequence can be added to the 5' untranslated region or the coding
sequence of the partial coding sequence to increase the amount of the mature message that
15 accumulates in the cytosol. Use of maize introns Adh1-S intron 1, 2, and 6, the Bronze-1
intron are known in the art. See generally, *The Maize Handbook*, Chapter 116, Freeling
and Walbot, Eds., Springer, New York (1994).

The vector comprising the sequences from a protease inhibitor nucleic acid will
typically comprise a marker gene which confers a selectable phenotype on plant cells.
20 Usually, the selectable marker gene will encode antibiotic resistance, with suitable genes
including genes coding for resistance to the antibiotic spectinomycin (e.g., the *aada* gene),
the streptomycin phosphotransferase (SPT) gene coding for streptomycin resistance, the
neomycin phosphotransferase (NPTII) gene encoding kanamycin or geneticin resistance,
the hygromycin phosphotransferase (HPT) gene coding for hygromycin resistance, genes
25 coding for resistance to herbicides which act to inhibit the action of acetolactate synthase
(ALS), in particular the sulfonylurea-type herbicides (e.g., the acetolactate synthase
(ALS) gene containing mutations leading to such resistance in particular the S4 and/or
Hra mutations), genes coding for resistance to herbicides which act to inhibit action of
glutamine synthase, such as phosphinothricin or basta (e.g., the *bar* gene), or other such
30 genes known in the art. The *bar* gene encodes resistance to the herbicide basta, the *nptII*
gene encodes resistance to the antibiotics kanamycin and geneticin, and the ALS gene
encodes resistance to the herbicide chlorsulfuron.

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Typical vectors useful for expression of genes in higher plants are well known in the art and include vectors derived from the tumor-inducing (Ti) plasmid of *Agrobacterium tumefaciens* described by Rogers *et al.*, Meth. In Enzymol., 153:253-277 (1987). These vectors are plant integrating vectors in that on transformation, the vectors
5 integrate a portion of vector DNA into the genome of the host plant. Exemplary *A. tumefaciens* vectors useful herein are plasmids pKYLX6 and pKYLX7 of Schardl *et al.*, Gene, 61:1-11 (1987) and Berger *et al.*, Proc. Natl. Acad. Sci. U.S.A., 86:8402-8406 (1989). Another useful vector herein is plasmid pBI101.2 that is available from Clontech Laboratories, Inc. (Palo
10 Alto, CA).

The protease inhibitor polynucleotide of the present invention can be expressed in either sense or anti-sense orientation as desired.

Protease inhibitor Proteins

The isolated protease inhibitor proteins of the present invention comprise a
15 protease inhibitor polypeptide having at least 10 amino acids encoded by any one of the protease inhibitor polynucleotides as discussed more fully, *supra*, or polypeptides which are conservatively modified variants thereof. Exemplary protease inhibitor polypeptide sequences are provided in SEQ ID NOS: 2,4,6,8,10 and 12. The protease inhibitor proteins of the present invention or variants thereof can comprise any number of
20 contiguous amino acid residues from a protease inhibitor protein, wherein that number is selected from the group of integers consisting of from 10 to the number of residues in a full-length protease inhibitor polypeptide. Optionally, this subsequence of contiguous amino acids is at least 15, 20, 25, 30, 35, or 40 amino acids in length, often at least 50, 60, 70, 80, or 90 amino acids in length. Further, the number of such subsequences can be any
25 integer selected from the group consisting of from 1 to 20, such as 2, 3, 4, or 5.

As those of skill will appreciate, the present invention includes protease inhibitor polypeptides with less inhibitory activity. Less inhibitory protease inhibitor polypeptides have an inhibitory activity at least 20%, 30%, or 40%, and preferably at least 50% or 60%, below that of the native (non-synthetic), endogenous protease inhibitor polypeptide.

30 A preferred immunoassay is a competitive immunoassay as discussed, *infra*.
Thus, the protease inhibitor proteins can be employed as immunogens for constructing

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such exemplary utilities as

may express a protease
bacteria, yeast, insect,
protein in a non-natural
environment, because they have been

able in the numerous
coding protease inhibitor
known for the expression

st, insect cell lines, plant
explained briefly below,
ressed in these eukaryotic
cells, as discussed *infra*,
ins of the instant

l to the instant invention;
available. As newer
y may be directly applied.
o insert a DNA sequence
translation of the sequence
d which provides for

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A. Plant Transformation

A DNA sequence coding for the desired protease inhibitor polynucleotide, for example a cDNA or a genomic sequence encoding a full length protein, will be used to
5 construct a recombinant expression cassette which can be introduced into the desired plant.

Isolated nucleic acids of the present invention can be introduced into plants according to techniques known in the art. Generally, recombinant expression cassettes as described above and suitable for transformation of plant cells are prepared. Techniques
10 for transforming a wide variety of higher plant species are well known and described in the technical, scientific, and patent literature. See, for example, Weising *et al.*, Ann. Rev. Genet. 22: 421-477 (1988). For example, the DNA construct may be introduced directly into the genomic DNA of the plant cell using techniques such as electroporation, PEG
15 poration, particle bombardment, silicon fiber delivery, or microinjection of plant cell protoplasts or embryogenic callus. Alternatively, the DNA constructs may be combined with suitable T-DNA flanking regions and introduced into a conventional *Agrobacterium tumefaciens* host vector. The virulence functions of the *Agrobacterium tumefaciens* host will direct the insertion of the construct and adjacent marker into the plant cell DNA when the cell is infected by the bacteria.

20 The introduction of DNA constructs using polyethylene glycol precipitation is described in Paszkowski *et al.*, Embo J. 3: 2717-2722 (1984). Electroporation techniques are described in Fromm *et al.*, Proc. Natl. Acad. Sci. 82: 5824 (1985). Ballistic transformation techniques are described in Klein *et al.*, Nature 327: 70-73 (1987). *Agrobacterium tumefaciens*-mediated transformation techniques are well described in the
25 scientific literature. See, for example Horsch *et al.*, Science 233: 496-498 (1984), and Fraley *et al.*, Proc. Natl. Acad. Sci. 80: 4803 (1983). Although *Agrobacterium* is useful primarily in dicots, certain monocots can be transformed by *Agrobacterium*. For instance, *Agrobacterium* transformation of maize is described in U.S. Patent No. 5,550,318.

Other methods of transfection or transformation include (1) *Agrobacterium*
30 *rhizogenes*-mediated transformation (see, e.g., Lichtenstein and Fuller In: Genetic Engineering, vol. 6, PWJ Rigby, Ed., London, Academic Press, 1987; and Lichtenstein, C. P., and Draper, J., In: DNA Cloning, Vol. II, D. M. Glover, Ed., Oxford, IRI Press,

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1985), Application PCT/US87/02512 (WO 88/02405 published Apr. 7, 1988) describes the use of *A. rhizogenes* strain A4 and its Ri plasmid along with *A. tumefaciens* vectors pARC8 or pARC16 (2) liposome-mediated DNA uptake (see, e.g., Freeman *et al.*, Plant Cell Physiol. 25: 1353, 1984), (3) the vortexing method (see, e.g., Kindle, *Proc. Natl. Acad. Sci.*, USA 87: 1228, (1990).

DNA can also be introduced into plants by direct DNA transfer into pollen as described by Zhou *et al.*, Methods in Enzymology, 101:433 (1983); D. Hess, Intern Rev. Cytol., 107:367 (1987); Luo *et al.*, Plant Mol. Biol. Reporter, 6:165 (1988). Expression of polypeptide coding genes can be obtained by injection of the DNA into reproductive organs of a plant as described by Pena *et al.*, Nature, 325:274 (1987). DNA can also be injected directly into the cells of immature embryos and the rehydration of desiccated embryos as described by Neuhaus *et al.*, Theor. Appl. Genet., 75:30 (1987); and Benbrook *et al.*, in Proceedings Bio Expo 1986, Butterworth, Stoneham, Mass., pp. 27-54 (1986). A variety of plant viruses that can be employed as vectors are known in the art and include cauliflower mosaic virus (CaMV), geminivirus, brome mosaic virus, and tobacco mosaic virus.

Synthesis of Proteins

Protease inhibitor proteins of the present invention can be constructed using non-cellular synthetic methods. Solid phase synthesis of protease inhibitor proteins of less than about 50 amino acids in length may be accomplished by attaching the C-terminal amino acid of the sequence to an insoluble support followed by sequential addition of the remaining amino acids in the sequence. Techniques for solid phase synthesis are described by Barany and Merrifield, Solid-Phase Peptide Synthesis, pp. 3-284 in *The Peptides: Analysis, Synthesis, Biology. Vol. 2: Special Methods in Peptide Synthesis, Part A.*; Merrifield, *et al.*, *J. Am. Chem. Soc.* 85: 2149-2156 (1963), and Stewart *et al.*, *Solid Phase Peptide Synthesis, 2nd ed.*, Pierce Chem. Co., Rockford, Ill. (1984). Also, the compounds can be synthesized on an applied Biosystems model 431a peptide synthesizer using fastmoc™ chemistry involving hbtu [2-(1h-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate, as published by Rao, *et al.*, *Int. J. Pep. Prot. Res.*; Vol. 40; pp. 508-515; (1992); incorporated herein in its entirety by reference. Peptides can be cleaved following standard protocols and purified by reverse phase

5 Methods of forming peptide bonds by activation of a carboxy terminal end (e.g., by the use of the coupling reagent N,N'-dicyclohexylcarbodiimide)) is known to those of skill.

The protease inhibitor proteins of the present invention may be purified by standard techniques well known to those of skill in the art. Recombinantly produced protease inhibitor proteins can be directly expressed or expressed as a fusion protein. The recombinant protease inhibitor protein is purified by a combination of cell lysis (e.g., sonication, French press) and affinity chromatography. For fusion products, subsequent digestion of the fusion protein with an appropriate proteolytic enzyme releases the desired recombinant protease inhibitor protein.

30 Transgenic Plant Regeneration

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genotype and thus the desired protease inhibitor content and/or composition phenotype. Such regeneration techniques often rely on manipulation of certain phytohormones in a tissue culture growth medium, typically relying on a biocide and/or herbicide marker which has been introduced together with the protease inhibitor polynucleotide.

5 Plants cells transformed with a plant expression vector can be regenerated, e.g., from single cells, callus tissue or leaf discs according to standard plant tissue culture techniques. It is well known in the art that various cells, tissues, and organs from almost any plant can be successfully cultured to regenerate an entire plant. Plant regeneration from cultured protoplasts is described in
10 Evans *et al.*, *Protoplasts Isolation and Culture, Handbook of Plant Cell Culture*, Macmillan Publishing Company, New York, pp. 124-176 (1983); and Binding, *Regeneration of Plants, Plant Protoplasts*, CRC Press, Boca Raton, pp. 21-73 (1985).

 The regeneration of plants containing the foreign gene introduced by *Agrobacterium* from leaf explants can be achieved as described by Horsch *et al.*,
15 Science, 227:1229-1231 (1985)

 Regeneration can also be obtained from plant callus, explants, organs, or parts thereof. Such regeneration techniques are described generally in Klee *et al.*, Ann. Rev. of Plant Phys. 38: 467-486 (1987) For maize cell culture and regeneration see generally, *The Maize Handbook*, Freeling and Walbot, Eds., Springer, New York (1994); *Corn and Corn*
20 *Improvement*, 3rd edition, Sprague and Dudley Eds., American Society of Agronomy, Madison, Wisconsin (1988).

 One of skill will recognize that after the recombinant expression cassette is stably incorporated in transgenic plants and confirmed to be operable, it can be introduced into other plants by sexual crossing. Any of a number of standard breeding techniques can be
25 used, depending upon the species to be crossed.

 In vegetatively propagated crops, mature transgenic plants can be propagated by the taking of cuttings or by tissue culture techniques to produce multiple identical plants. Selection of desirable transgenics is made and new varieties are obtained and propagated vegetatively for commercial use. In seed propagated crops, mature transgenic plants can
30 be self crossed to produce a homozygous inbred plant. The inbred plant produces seed containing the newly introduced heterologous nucleic acid. These seeds can be grown to

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produce plants that would produce the selected phenotype, (e.g., altered protease inhibitor content or composition).

Parts obtained from the regenerated plant, such as flowers, seeds, leaves, branches, fruit, and the like are included in the invention, provided that these

5 parts comprise cells comprising the isolated nucleic acid of the present invention. Progeny and variants, and mutants of the regenerated plants are also included within the scope of the invention, provided that these parts comprise the introduced nucleic acid sequences.

Transgenic plants expressing the selectable marker can be screened for

10 transmission of the protease inhibitor nucleic acid of the present invention by, for example, standard immunoblot and DNA detection techniques. Transgenic lines are also typically evaluated on levels of expression of the heterologous nucleic acid. Expression at the RNA level can be determined initially to identify and quantitate expression-positive plants. Standard techniques for RNA analysis can be employed and include PCR

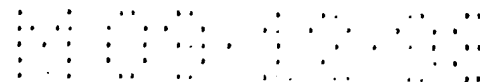
15 amplification assays using oligonucleotide primers designed to amplify only the heterologous RNA templates and solution hybridization assays using heterologous nucleic acid-specific probes. The RNA-positive plants can then analyzed for protein expression by Western immunoblot analysis using the protease inhibitor specific antibodies of the present invention. In addition, *in situ* hybridization and immunocytochemistry according

20 to standard protocols can be done using heterologous nucleic acid specific polynucleotide probes and antibodies, respectively, to localize sites of expression within transgenic tissue. Generally, a number of transgenic lines are usually screened for the incorporated nucleic acid to identify and select plants with the most appropriate expression profiles.

A preferred embodiment is a transgenic plant that is homozygous for the added

25 heterologous nucleic acid; i.e., a transgenic plant that contains two added nucleic acid sequences, one gene at the same locus on each chromosome of a chromosome pair. A homozygous transgenic plant can be obtained by sexually mating (selfing) a heterozygous transgenic plant that contains a single added heterologous nucleic acid, germinating some of the seed produced and analyzing the resulting plants produced for altered activity

30 relative to a control plant (i.e., native, non-transgenic). Back-crossing to a parental plant and out-crossing with a non- transgenic plant are also contemplated.

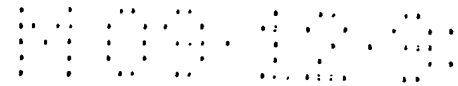


Protein structure and amino acid substitution

It can be difficult to predict the ultimate effect of substitution on the tertiary structure and folding of the protein. Both tertiary structure and folding are critical to the stability and adequate expression of the protein in vivo. It is critical to undertake analysis and functional modeling of the wild type compound to determine whether substitutions can be made without disrupting biological activity.

The biological activity of a protein is dictated by its three dimensional structure which is intrinsically related to the folding of the protein. The folding of a protein into its functional domains is a direct consequence of the primary amino acid sequence. While it is true that many proteins tolerate amino acid changes without affecting the folding or function of the protein, there is no a priori method of predicting which amino acid may be substituted or deleted without affecting the folding pathway. Each protein is unique and the folding process is necessarily an experimental determination. As has been concluded by Zabin et al., ("Approaches to Predicting Effects of Single Amino Acid Substitutions on the Function of a Protein"; Biochemistry; Vol. 30; pp. 6230-6240; 1991), neither the frequency of exchange of amino acids between homologous proteins nor any other measure of the properties of the amino acids are particularly useful by themselves in predicting whether a protein with an amino acid substitution will be functional. The scientific literature is replete with examples where seemingly conservative substitutions have resulted in major perturbations of structure and activity and vice versa, see e.g.; Summers, et al., "A Conservative Amino Acid Substitution, Arginine for Lysine, Abolishes Export of a Hybrid Protein in E. Coli," J. Biol. Chem., Vol. 264, pp. 20082-20088, (1989); Ringe, D., "The Sheep in-Wolf's Clothing" Nature, Vol. 339, pp. 658-659, (1989); Hirabayashi et al., "Effect of Amino Acid Substitution by Site-directed Mutagenesis on the Carbohydrate Recognition and Stability of Human 14-kDa β -galactoside-binding Lectin," J. Biol. Chem., Vol. 266, pp. 23648-23653, (1991); and van Eijdsden, et al., "Mutational Analysis of Pea Lectin: Substitution of Asn125 for Asp in the Monosachharide-binding Site Eliminates Mannose/Glucose -binding Activity," Plant Mol. Biol., Vol. 20, pp. 1049-1058 (1992); all incorporated herein in their entirety by reference.

The 3D structure of many proteins, including enzymes and protein inhibitors such as the barley chymotrypsin inhibitor has been solved. The three dimensional structure of a



truncated fragment of CI-2 (with 65 residues) that is missing the N-terminal 18 residues has been determined by x-ray crystallography as well as by NMR spectroscopy (McPhalen, et al., Biochemistry; Vol. 26; pp. 261-269; (1987); and Clore, et al., Protein Eng.; Vol. 1, pp. 313-318; (1987)). In the wild type CI-2 the first 18 residues do not
5 assume any ordered conformation and also do not contribute to the structural integrity of the molecule (see e.g. Kjaer, et al., Carlsberg Res. Commun.; Vol. 53; pp. 327-354; (1987); incorporated herein in its entirety by reference), This polypeptide is found in the endosperm of grain and is isolated as an 83 residue protein with no disulfide bridges. See e.g. Jonassen, I., Carlsberg Res. Commun.; Vol. 45; pp. 47-48; (1980); and Svendsen, I.,
10 et al., Carlsberg Res. Commun.; Vol. 45; pp. 79-85; (1980). The 3D structure of CI-2 has been determined. See McPhalen, et al., 1987; incorporated herein in its entirety by reference. CI-2 is predominantly a β -sheet protein, devoid of disulfide bonds and containing a wide loop of approximately 18 residues (residue 53-70 in the CI-2 molecule) in the extended conformation. This is the reactive site loop that contains a methionine
15 residue at position 59 which confers the property of chymotrypsin inhibition. A constrained peptide containing these residues has been synthesized and shown to retain full chymotrypsin inhibitory activity. See Leatherbarrow, et al., Biochem., Vol. 30, pp. 10717-10721 (1991). In the absence of any disulfide bonds, the integrity of the reactive site loop is maintained by strong hydrogen bond interactions between Glu60 \rightarrow Arg65
20 and Thr58 \rightarrow Arg67. Mutants of CI-2 in which Thr58 and Glu60 have been replaced with Ala are not only less stable proteins but also have little or no protease inhibitory activity. See Jackson, et al., Biochem., Vol. 33, pp. 13880-13887 (1994); and Jandu, et al., Biochem., Vol. 33, pp. 6264-6269 (1990). These studies have demonstrated that the reactive site loop is a key structural feature essential for the function of protease
25 inhibition.

Molecular Markers

The present invention provides a method of genotyping a plant comprising a
30 protease inhibitor polynucleotide. Preferably, the plant is a monocot, such as maize or sorghum. Genotyping provides a means of distinguishing homologs of a chromosome pair and can be used to differentiate segregants in a plant population.

P A T E N T

Molecular marker methods can be used for phylogenetic studies, characterizing genetic relationships among crop varieties, identifying crosses or somatic hybrids, localizing chromosomal segments affecting monogenic traits, map based cloning, and the study of quantitative inheritance. See, e.g., *Plant Molecular Biology: A Laboratory Manual*, Chapter 7, Clark, Ed., Springer-Verlag, Berlin (1997). For molecular marker methods, see generally, *The DNA Revolution* by Andrew H. Paterson 1996 (Chapter 2) in: *Genome Mapping in Plants* (ed. Andrew H. Paterson) by Academic Press/R. G. Landis Company, Austin, Texas, pp.7-21.

10 Detection of Protease Inhibitor Nucleic Acids

The present invention further provides methods for detecting protease inhibitor polynucleotides of the present invention in a nucleic acid sample suspected of comprising a protease inhibitor polynucleotide, such as a plant cell lysate, particularly a lysate of corn. In some embodiments, a protease inhibitor gene or portion thereof can be amplified prior to the step of contacting the nucleic acid sample with a protease inhibitor polynucleotide. The nucleic acid sample is contacted with the protease inhibitor polynucleotide to form a hybridization complex. The protease inhibitor polynucleotide hybridizes under stringent conditions to a gene encoding a protease inhibitor polypeptide. Formation of the hybridization complex is used to detect a gene encoding a protease inhibitor polypeptide in the nucleic acid sample. Those of skill will appreciate that an isolated nucleic acid comprising a protease inhibitor polynucleotide should lack cross-hybridizing sequences with non-protease inhibitor genes that would yield a false positive result.

Detection of the hybridization complex can be achieved using any number of well known methods. For example, the nucleic acid sample, or a portion thereof, may be assayed by hybridization formats including but not limited to, solution phase, solid phase, mixed phase, or *in situ* hybridization assays.

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Protease Inhibitor Protein Immunoassays



Means of detecting the protease inhibitor proteins of the present invention are not critical aspects of the present invention. In a preferred embodiment, the protease inhibitor proteins are detected and/or quantified using any of a number of well recognized immunological binding assays (*see, e.g.*, U.S. Patents 4,366,241; 4,376,110; 4,517,288; and 4,837,168). For a review of the general immunoassays, see also *Methods in Cell Biology*, Vol. 37: *Antibodies in Cell Biology*, Asai, Ed., Academic Press, Inc. New York (1993); *Basic and Clinical Immunology* 7th Edition, Stites & Terr, Eds. (1991).

D. Other Assay Formats

In a particularly preferred embodiment, Western blot (immunoblot) analysis is used to detect and quantify the presence of protease inhibitor protein in the sample. The technique generally comprises separating sample proteins by gel electrophoresis on the basis of molecular weight, transferring the separated proteins to a suitable solid support, (such as a nitrocellulose filter, a nylon filter, or derivatized nylon filter), and incubating the sample with the antibodies that specifically bind protease inhibitor protein. The anti-protease inhibitor protein antibodies specifically bind to protease inhibitor protein on the solid support. These antibodies may be directly labeled or alternatively may be subsequently detected using labeled antibodies (*e.g.*, labeled sheep anti-mouse antibodies) that specifically bind to the anti-protease inhibitor protein.

E. Quantification of Protease inhibitor Proteins.

Protease inhibitor proteins may be detected and quantified by any of a number of means well known to those of skill in the art. These include analytic biochemical methods such as electrophoresis, capillary electrophoresis, high performance liquid chromatography (HPLC), thin layer chromatography (TLC), hyperdiffusion chromatography, and the like, and various immunological methods such as fluid or gel precipitin reactions, immunodiffusion (single or double), immunoelectrophoresis, radioimmunoassays (RIAs), enzyme-linked immunosorbent assays (ELISAs), immunofluorescent assays, and the like.

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Example 1: Isolation of DNA Coding for Protease inhibitor Protein from *Zea mays* or other plant library

The polynucleotides having DNA sequences given in SEQ ID Nos: 15, 17, 19, 21, and 23
5 were obtained from the sequencing of cDNA clones prepared from maize.

SEQ ID NO 15 is a contig comprised of 28 cDNA clones. 20 of the cDNA clones were
from libraries prepared from leaves treated with jasmonic acid. One was from a root
library. Four were from libraries prepared from corn rootworm-infested roots. One was
from a tassel library. One was from a library prepared from seedlings recovering from
10 heat shock. One was from a shoot culture library.

SEQ ID NO 17 is a contig comprised of two cDNA clones. One was from a jasmonic acid
treated leaf library. The other was from an induced resistance leaf library.

SEQ ID NO 19 is a contig comprised of two cDNA clones. One was from a germinating
maize seedling library. The other was from jasmonic acid treated leaf library.

15 SEQ ID NO 21 is a contig comprised of 4 cDNA clones. All four were from libraries
prepared from jasmonic acid treated leaves.

SEQ ID NO 5 is a contig comprised of two cDNA clones. One was from a library
prepared from silks, 24 hours post pollination. The other was from a library prepared
from root tips less than 5 mm in length.

20

One skilled in the art could apply these same methods to other plant nucleotide containing
libraries.

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Example 2: Engineering BHL for nutritional enhancement

Wild type CI-2 (from barley) contains 49.4% essential amino acids (41/83) and
9.6% lysine (8/83). Using the strategies outlined below, six different BHL variants with
30 increasing amounts of lysine have been proposed. The lysine percentages are 21.5%,
24.1%, 23.1%, and 25.3%, for BHL-1, BHL-1N, BHL-2, BHL-2N, BHL-3, and BHL-3N,
respectively. Construct BHL-1N contains the same eight substitutions as BHL-1, plus
lysine substitutions in the 18 additional amino acid residues in the amino terminal
region. BHL-2 is the same as BHL-1 but with changes of amino acid residues 40 and 42

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to Ala and amino acid residue 47 to lysine. Construct BHL-2N contains the same 11 substitutions as BHL-2, plus four lysine substitutions in the 18 additional amino acid residues in the amino terminal region. BHL-3 is the same as BHL-2 except that residues 40 and 42 are changed to Gly and His, respectively. Construct BHL-3N contains the same 11 substitutions as BHL-3, plus the four lysine substitutions in 18 additional amino acid residues in the amino terminal region. One skilled in the art will realize that essential and non-wild-type amino acid residue substitutions will be tolerated at both the same positions substituted with lysine, and at other positions.

The active site loop region encompasses an extended loop region from about amino acid residue 53 to about amino acid residue 70. Destabilization of the reactive loop was achieved by substituting the non-wild type amino acids residues at about positions 53 to about 70. Amino acid residues were changed by primer mutagenesis. Preferably, the following mutations are made: Arg62 → Lys62, Arg65 → Lys65, Arg67 → Lys67, Thr58 → Ala58 or Gly58, Met59 → Lys59, and Glu60 → Ala60 or His60. However, it will be readily apparent to one skilled in the art that functionally equivalent substitutions to those described above will also be effective in the present invention.

In a preferred embodiment of the present invention, the present protein has both elevated essential amino acid content and reduced protease inhibitor activity.

Modification in the area by amino acid substitution or other means, destroys the hydrogen bonding and changes or reduces the protease inhibitor activity of BHL. Substitution of amino acid residues threonine, at position 58, and glutamic acid, at position 60, with glycine and histidine, respectively, resulted in a protein with lowered protease inhibitor activity. Residue 59 is a critical residue in modifying protease inhibitor activity and changing specificity. When this residue was changed to a lysine, the protease inhibition specificity was changed from a chymotrypsin inhibitor to a trypsin inhibitor.

The present invention provides for the creation of a nutritionally enhanced feed from WT CI-2 through at least one lysine substitution of residues 1,18,11,17,19,34,41,56,59,62,67 and 73 (long versions BHL-1N, 2N, 3N) plus residue 67 in BH2-2N and BH2-3N. Lysine substitutions in BHL-1,2 and 3 are at amino acid residues 1,16,23,41,44,49 and 55, plus residue 47 in BHL-2 and BHL-3.

Example 3- Construction of Expression Cassettes

BHL-1

20 Oligonucleotide and primer sequences (5' to 3'):
N4394

25
51 AAGCCAAGAA GGTGATCCTG AAGGACAAGC CAGAGGCGCA
AATCATAGTT

30
N4395

35
51 TTCTTGGCTT TCTCCACCGA TTTCCCCACC AACTCCGGCC
ACTCTGTCTT

101 CAGCTT

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60 61 62 63 64 65 66 67 68 69 70 71 72 73 74 75 76 77 78 79 80 81 82 83 84 85 86 87 88 89 90 91 92 93 94 95 96 97 98 99 100

N4396

1 CGGTTGGTAC AAAGGTGACG AAGGAATATA AGATCGACCG
5 CGTCAAGCTC

51 TTTGTGGATA AAAAGGACAA CATCGCGCAG GTCCCCAGGG TCGG

10 N4397

1 CTAGCCGACC CTGGGGACCT GCGCGATGTT GTCCTTTTAA
TCCACAAAGA

15 51 GCTTGACGCG GTCGATCTTA TATTCCTTCG TCACCTTTGT AC

N5045

20 1 GTACTAGTCA TGAAGCTGAA GACAGA

N5046

25 1 GAGAAGCTTG CTAGCCGACC CTGGGGAC

b. BHL-2: The BHL-2 construct insert corresponds to SEQ ID NO 3, plus start and stop codons. An overlap PCR strategy was used to make the BHL-2 construct. PWO polymerase from Boehringer-Mannheim was used for all PCR reactions. The primers were
30 chosen to change 3 amino acids in the BHL-1 active site loop region, and to create unique *Age*I and *Hind* III restriction sites flanking the active site loop, to facilitate loop replacement in future constructs. A unique *Rca* I site (compatible with *Nco* I) was included at the 5' end, and a unique *Xho* I site was included at the 3' end. The overlap PCR was done as follows: PCR was done with primers N13561 and N13564, using the
35 BHL-1 construct as template. A separate PCR was done with primers N13563 and N13562, again using the BHL-1 construct as template. The products from both reactions were gel purified and combined. Primer N13565, which overlapped regions on both of the PCR products, was then added and another PCR was done to generate the full-length insert. The resulting product was amplified by another PCR with primers N13561 and
40 N13562. It was subsequently suspected that a deletion was present in N13562 that caused a frameshift near the 3' end of the PCR product. To avoid this frameshift problem, a final

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PCR reaction was done with primers N13562 and N13905. The final PCR product was digested with *Rca* I and *Xho* I, and then ligated into the *Nco* I and *Xho* I sites of pET 28b.

Note: Some primers had 6-oligonucleotide extensions to improve restriction digestion efficiency.

5

Primer sequences (5' TO 3'):

N13561

1 TTTTTTTCATGAAGCTGAAGACA

N13562 (as ordered)

10 1 TTTTTTCTCGAGGCTAGCCGACCCTGGGGA

N13563

1 ATCGACAAGGTCAAGCTTTTTGTGGATAAAAAGGA

N13564

1 CACCTTTGTACCAACCGGTAGAACTATGATTTGCGC

15 N13565

1 GTTGGTACAAAGGTGGCGAAGGCCTATAAGATCGACAAGGTCAAG

N13905

1 TTTTTTCTCGAGGCTAGCCGACCCTGGGGACCTGCGCTA

20 c. BHL-3: The BHL-3 construct insert corresponds to SEQ ID NO 5, plus start and stop codons. The BHL-2 construct was digested with *Age* I and *Hind* III, and the region between these sites was removed by gel purification. Oligonucleotide pairs, N14471 and N14472, were annealed to make a double stranded DNA molecule with overhangs compatible with *Age* I and *Hind* III restriction sites. The annealed product was ligated
25 into the *Age* I and *Hind* III sites of the digested BHL-2 construct to yield the BHL-3 construct.

Oligonucleotide Primer sequences (5' to 3'):

N14471

1 CCGGTTGGTACAAAGGTGGGTAAGCATTATAAGATCGACAAGGTCA

30 N14472

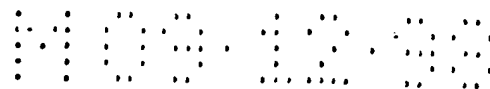
1 AGCTTGACCTTGTCGATCTTATAATGCTTACCCACCTTTGTACCAA

d. BHL-1N, BHL-2N, and BHL-3N

The BHL-1N, BHL-2N, and BHL-3N construct inserts correspond to SEQ ID No 9, SEQ ID NO 11, and SEQ ID NO 7, respectively, plus start and stop codons. Three separate
35 PCR reactions were done with either the BHL-1, BHL-2, or BHL-3 constructs as template. The primers for these reactions were N13771 and N13905. The resulting PCR products were digested with *Rca* I and *Xho* I and ligated into the *Nco* I and *Xho* I sites of pET 28b to yield the BHL-1N, BHL-2N, and BHL-3N constructs.

Primer sequences (5' to 3'):

40 N13771



1
TTTTTTTCATGAAGTCGGTGGAGAAGAAACCGAAGGGTGTGAAGACAGG
50 TGCGGGTGACAAGCATAAGCTGAAGACAGAGTG
N13905 (already provided in BHL-2 description)

5 BHL-1N is an 83 residue polypeptide in which residues 1,8,11, and 17 were also replaced with lysine. The resulting compound has the protein sequence indicated in Sequence I.D. No.10.

BHL-2N is an 83 residue polypeptide in which residues 1,8,11, and 17 were also replaced with lysine. The resulting compound has the protein sequence indicated in
10 Sequence I.D. No.12.

BHL-3N is an 83 residue polypeptide in which residues 1,8,11, and 17 were also replaced with lysine. The resulting compound has the protein sequence indicated in Sequence I.D. No.8.

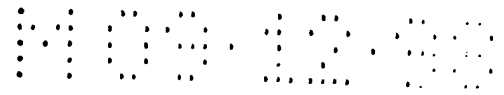
15 Example 3 - Expression of BHL-1 in *E. coli*

Expression in E. coli

BHL-1, BHL-2, BHL-3, BHL-3N, and the truncated wild-type CI-2 (residues 19 through 65 of SEQ ID NO. 14) were expressed in *E coli* using materials and methods from Novagen, Inc. The Novagen expression vector pET-28 was used (pET-28a for WT CI-2
20 and BHL-1, and pET-28b for the other proteins). *Ecoli* strains BL21(DE-3) or BL21(DE-3)pLysS were used. Cultures were typically grown until an OD at 600 nm of 0.8 to 1.0, and then induced with 1 mM IPTG and grown another 2.5 to 5 hours before harvesting. Induction at an OD as low as 0.4 was also done successfully. Growth temperatures of 37 degrees centigrade and 30 degrees centigrade were both used successfully. The media
25 used was 2xYT plus the appropriate antibiotic at the concentration recommended in the Novagen manual.

Purification

a. WT CI-2 (truncated)-- Lysis buffer was 50 mM Tris-HCl, pH 8.0, 1 mM EDTA,
30 150 mM NaCl. The protein was precipitated with 70% ammonium sulfate. The pellet was dissolved and dialyzed against 50 mM Tris-HCl, pH 8.6. The protein was loaded onto a Hi-Trap Q column, and the unbound fraction was collected and precipitated in 70% ammonium sulfate. The pellet was dissolved in 50 mM sodium phosphate, pH 7.0, 200



mM NaCl, and fractionated on a Superdex-75 26/60 gel filtration column. Fractions were pooled and concentrated.

b. BHL-1--Lysis buffer was 50 mM sodium phosphate, pH 7.0, 1 mM EDTA.

The protein was loaded onto an SP Sepharose FF 16/10 column, washed with 150 mM
5 NaCl in 50 mM sodium phosphate, pH 7.0, and then eluted with an NaCl gradient in 50 mM sodium phosphate. BHL-1 eluted at approximately 200 mM NaCl. Fractions were pooled and concentrated.

c. BHL-2, BHL-3, and BHL-3N--Lysis buffer was 50 mM Hepes, pH 8.0, 2mM
10 EDTA, 0.1% Triton X-100, and 0.5 mg/ml lysozyme. The protein was loaded onto an SP-Sepharose cation exchange column (typically a 5 to 10 ml size), washed with 150 mM NaCl in 50 mM sodium phosphate, pH 7.0, and eluted with 500 mM NaCl in 50 mM sodium phosphate, pH 7.0. The protein was concentrated and then subjected to Superdex-75 gel filtration chromatography twice.

d. BHL-1--Lysis buffer was 50 mM sodium phosphate, pH 7.0, 1 mM EDTA.

15 The protein was loaded onto an SP Sepharose FF 16/10 column, washed with 150 mM NaCl in 50 mM sodium phosphate, pH 7.0, and then eluted with an NaCl gradient in 50 mM sodium phosphate. BHL-1 eluted at approximately 200 mM NaCl. Fractions were pooled and concentrated.

e. BHL-2, BHL-3, and BHL-3N--Lysis buffer was 50 mM Hepes, pH 8.0, 2mM
20 EDTA, 0.1% Triton X-100, and 0.5 mg/ml lysozyme. The protein was loaded onto an SP-Sepharose cation exchange column (typically a 5 to 10 ml size), washed with 150 mM NaCl in 50 mM sodium phosphate, pH 7.0, and eluted with 500 mM NaCl in 50 mM sodium phosphate, pH 7.0. The protein was concentrated and then subjected to Superdex-75 gel filtration chromatography twice.

25 4. Storage

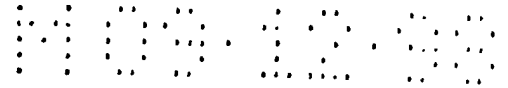
The purified proteins were stored long term by freezing in liquid nitrogen and keeping frozen at -70 degrees centigrade.

5. Verification of recombinant protein identity.

30 a. DNA sequencing--

The insert region of these pET 28 constructs was confirmed by DNA sequencing.

b. N-terminal protein sequencing --



100 µg of purified BHL-3 were digested with 1 µg of chymotrypsin (Sigma catalog # C-4129) for 30 min at 37 degrees centigrade in 50 mM sodium phosphate, pH 7.0. The resulting chymotryptic fragments were purified by reversed phase chromatography, using an acetonitrile gradient for elution. Three pure peaks were observed and were sent to the University of Michigan Medical School Protein Structure Facility for N-terminal sequencing (6 cycles). Peak 1 had an N-terminal sequence of val-asp-lys-lys-asp-asn. Peak 2 had an N-terminal sequence of lys-ile-asp-lys-val-lys. Peak 3 had an N-terminal sequence of met-lys-leu-lys-thr-glu. These results demonstrate that chymotrypsin cleaved BHL-3 after tyr-61 and phe-69. The N-terminal sequences all match exactly the BHL-3 expected sequence, assuming that the start methionine was largely retained in the recombinant protein. This experiment verifies that the protein we expressed in and purified from *E. coli* was BHL-3. Furthermore, SDS-PAGE analysis with 16.5% Tris-Tricine precast gels from Biorad showed a similar mobility of BHL-1 and BHL-2 with the confirmed BHL-3 protein, as would be expected because BHL-1 and BHL-2 have molecular masses very similar to that of BHL-3.

160 µg of BHL-3N were digested with 1.6 µg pepsin overnight, and the resulting peptic fragments were purified by reversed phase chromatography. Five of the resulting peaks were sent to the Iowa State University Protein Facility for N-terminal sequencing through four cycles. The N-terminal sequences of the 5 peaks were: val-gly-lys-ser, phe-val-asp-lys, pro-val-gly-thr, met-lys-ser-val, and ile-ile-val-leu, all of which exactly match the expected BHL-3N sequence, assuming that the start methionine was largely retained in this recombinant protein. This experiment verifies that the protein we expressed in and purified from *E. coli* was BHL-3N.

c. Protease inhibition--

The obvious protease inhibitory activity observed for BHL-1 and for the wild-type protein are further evidence that we have purified the expected proteins from *E. coli*. The details of these protease inhibition experiments are described next.

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The following experiments utilized truncated wild type CI-2 as represented as nt. 55-249 in Seq. ID NO. 13 with addition of start and stop codons.

Example 5 - Protease Inhibition assays and Proteolytic Digests

5 a. Chymotrypsin

Protease activity was measured by an increase in absorbance at 405 nm.

Sigma Chymotrypsin type II (Bovine pancreas) Cat. # C-4129.

Substrate - Sigma cat. # 5-7388. N-Succinyl-Ala-Ala-Pro-phe-p nitro anilide or BHL protein used, 1 μ M chymotrypsin, 1mM substrate, 200 μ l volume

10 1 μ M BSA included in control (no CI-2, no BHL).

Preincubated 30 min 37° C., then added substrate to start and kept at 37° C.

Buffer 0.2M tris - HCl pH 8.0

Read Abs 405 nm - 30 min

Protease Activity - % of Control ABS. 405 nm

15

	Abs. At 405 nm			
	Rep. 1	Rep. 2	Mean (S.D.) Using % control data	
Control 1-value	0.350	0.299		
% control	100.0	100.0	100.0	
WT CI-2-value	.042	.018		
% control	12.0	6.0	9.0	(4.2)
BHL-1-value	.289	.274		
% control	82.6	91.6	87.1	(6.4)
BHL-2-value	.309	.318		
% control	88.3	106.4	97.4	(12.8)
BHL-3-value	.346	.315		
% control	98.9	105.4	102.2	(4.6)
BHL-3N-value	.318	.315		
% control	90.9	105.4	98.2	(10.3)

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b. Subtilisin

Subtilisin carlsberg from *Bacillus licheniformis* (Sigma cat. # P-5380)

Substrate and buffer same as for chymotrypsin exper. 200 ul reaction volume

1 um CI2 or BHL

5 1nM subtilisin

1mM Substrate

room temp (25° C)

30 min. preincubated then added substrate and read absorbance at 405nm

30 min. data used

10 1uM BSA used in control (no CI2 or BHL)

	Abs. At 405 nm			
	Rep. 1	Rep. 2	Mean (S.D.) Using % control data	
Control 1-value	2.171	1.834		
% control	100.0	100.0	100.0	
WT CI-2-value	.014	.002		
% control	0.6	0	0.3	(0.4)
BHL-1-value	.286	.295		
% control	13.2	16.1	14.7	(2.1)
BHL-2-value	1.692	1.569		
% control	77.9	85.6	81.8	(5.4)
BHL-3-value	7.056	1.960		
% control	94.7	106.9	100.8	(8.6)
BHL-3N-value	2.103	1.729		
% control	96.9	94.3	95.6	(1.8)

0571R-PCT

c. Trypsin

Bovine pancreas trypsin (Sigma cat #T-8919)

Substrate S-2222 (chromogenix): N-benzoyl-2-isoleuene-L-glutamyl-glycyl-L-arginine-p-nitroaniline

5 buffer: 50mM Tris pH 7.5, 2mM NaCl, 2mM CaCl₂, 0.005 % TritonX-100.

30 min. preincubation 25°, then added substrate and kept at 25°; these are 30 minute values.

1 mM substrate, 5uM CI-2 or BHL, 0.5nM trypsin, no BSA in control. 200 ul reaction volume

	Abs. At 405nm					Mean (S.D.) Using % Control Data
	Rep. 1	Rep. 2	Rep. 3	Rep. 4		
Control 1-value	.505	.533	.473	.391		
% control	100.0	100.0	100.0	100.0	100.0	
WT CI-2-value	.561	.533	.474	.420		
% control	111.1	100.0	100.2	107.4	104.7	(5.5)
BHL-1-value	.072	.096	.041	.057		
% control	14.3	18.0	8.7	14.6	13.9	(3.9)
BHL-2-value	.436	.481	.404	.405		
% control	86.3	90.2	85.4	103.5	91.4	(8.4)
BHL-3-value	.536	.557	.456	.430		
% control	106.1	104.5	96.4	110.0	104.3	(5.7)
BHL-3N-value	.542	.583	.490	.437		
% control	107.3	109.4	103.6	111.8	108.0	(3.5)

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d. Elastase

Porcine elastase Type IV (Sigma) Cat# E-0258

Substrate: Sigma S-4760 N-succinyl-ala-ala-ala-p-nitroanile

buffer: 0.2M Tris HCl pH 8.0 200 ul reactive volume 50nM elastase, 2 uM CI-2 or BHL;

5 1mM substrate

1uM BSA in control

15 min. preincub, 25°, then added substrate. Kept at 25°; 30 min. data

	Abs. At 405 nm			
	Rep. 1	Rep. 2	Mean (sp) Using % control data	
Control 1-value	1.416	1.461		
% control	100.0	100.0	100.0	
WT CI-2-value	.030	.049		
% control	2.1	3.4	2.8	(0.9)
BHL-1-value	1.519	1.459		
% control	107.3	99.9	103.6	(5.2)
BHL-2-value	1.558	1.509		
% control	110.0	103.3	106.7	(4.7)
BHL-3-value	1.587	1.493		
% control	112.1	102.2	107.2	(7.0)
BHL-3N-value	1.527	1.481		
% control	107.8	101.4	104.6	(4.5)

Protein	Chymotrypsin	Trypsin	Elastase	Subtilisin
WT CI-2	9.0	104.7	2.8	0.3
BHL-1	87.1	13.9	103.6	14.7
BHL-2	97.4	91.4	106.7	81.8
BHL-3	102.2	104.3	107.2	100.8
BHL-3N	98.2	108.0	104.6	95.6

Digestion by trypsin

Digestion by chymotrypsin.

Digestion in simulated gastric fluid.

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Simulated gastric fluid was prepared by dissolving 20 mg NaCl and 32 mg of pepsin in 70 µl of HCl plus enough water to make 10 ml. Porcine stomach pepsin (Sigma cat # P-6887) was used. 50 µl of 1 mg/ml BHL-3N or wild-type CI-2 protein were incubated with 250 µl simulated gastric fluid at 37 degrees centigrade. At 15 sec, 30 sec, 5 1 min, 5 min, and 30 min, 40 µl aliquots were removed to a stop solution consisting of 40 µl 2X Tris-Tricine SDS sample buffer (Biorad) that also contained 3 µl of 1 M Tris-HCl, pH 8.0 and 0.1 mg/ml pepstatin A (Boehringer-Mannheim cat # 60010). Digestion was assessed by 16.5% Tris-Tricine SDS-PAGE (precast gels from Biorad).

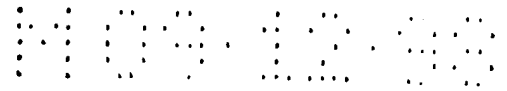
10 Both BHL-3N and wild-type CI-2 were digested in simulated gastric fluid in 15 seconds. This experiment suggests that our engineered proteins and even the wild-type protein would likely be digested into proteolytic fragments in the stomach of humans or monogastric animals.

15 *Digestion in simulated intestinal fluid.*

Simulated intestinal fluid was prepared by dissolving 68 mg of monobasic potassium phosphate in 2.5 ml of water, adding 1.9 ml of 0.2 N sodium hydroxide and 4 ml of water. Then 2.0 g porcine pancreatin (Sigma catalog # P-7545) was added and the resulting solution was adjusted with 0.2N sodium hydroxide to a pH of 7.5. Water was 20 added to make a final volume of 10 ml.

50 µg of BHL-3N or wild-type CI-2 protein in 50 µl were incubated with 250 µl simulated intestinal fluid at 37 degrees centigrade . At 15 sec, 30 sec, 1 min, 5 min, and 30 min, 40 µl aliquots were removed and added to 40 µl of a stop solution consisting of 25 2X Tris-Tricine SDS sample buffer (Biorad) containing 2 mM EDTA and 2mM phenylmethylsulfonyl fluoride (Sigma catalog # P-7626). Digestion was assessed by 16.5 % Tris-Tricine SDS-PAGE (precast gels form Biorad).

BHL-3N was digested by simulated intestinal fluid in 15 seconds. In contrast, 30 wild-type CI-2 was resistant to digestion for 30 minutes. This experiment shows that in the intestine of humans or monogastric animals, our engineered protein would likely be more digestible than the wild-type protein would be. These results are consistent with the



protease inhibition assays showing that BHL-3N was not an effective protease inhibitor. The inventive protein was digested in less than five minutes, less than one and less than 30 seconds.

5 *Digestion in simulated gastric fluid*

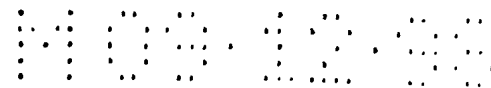
Simulated gastric fluid was prepared by dissolving 20 mg NaCl and 32 mg of pepsin in 70 µl of HCl plus enough water to make 10 ml. Porcine stomach pepsin (Sigma cat # P-6887) was used. 50 µl of 1 mg/ml BHL-3N or wild-type CI-2 were incubated with 250 µl simulated gastric fluid at 37 degrees centigrade. At 15 sec, 30 sec, 1 min, 5
10 min, and 30 min, 40 µl aliquots were removed to a stop solution consisting of 40 µl 2X Tris-Tricine SDS sample buffer (Biorad) that also contained 3 µl of 1 M Tris-HCl, pH 8.0 and 0.1 mg/ml pepstatin A (Boehringer-Mannheim cat # 60010). Digestion was assessed by 16.5% Tris-Tricine SDS-PAGE (precast gels from Biorad™).

Both BHL-3N and wild-type CI-2 were digested in simulated gastric fluid in 15
15 seconds. This experiment suggests that our engineered proteins and even the wild-type protein would likely be digested into proteolytic fragments in the stomach of humans or monogastric animals.

Digestion in simulated intestinal fluid.

20 Simulated intestinal fluid was prepared by dissolving 68 mg of monobasic potassium phosphate in 2.5 ml of water, adding 1.9 ml of 0.2 N sodium hydroxide and 4 ml of water. Then 2.0 g porcine pancreatin (Sigma catalog # P-7545) was added and the resulting solution was adjusted with 0.2N sodium hydroxide to a pH of 7.5. Water was added to make a final volume of 10 ml.

25 50 µl of 1mg/ml BHL-3N or wild-type CI-2 were incubated with 250 µl simulated intestinal fluid at 37 degrees centigrade . At 15 sec, 30 sec, 1 min, 5 min, and 30 min, 40 µl aliquots were removed and added to 40 µl of a stop solution consisting of 2X Tris-Tricine SDS sample buffer (Biorad) containing 2 mM EDTA and 2mM
30 phenylmethylsulfonyl fluoride (Sigma catalog # P-7626). Digestion was assessed by 16.5 % Tris-Tricine SDS-PAGE (precast gels form Biorad).



BHL-3N was digested by simulated intestinal fluid in 15 seconds. In contrast, wild-type CI-2 was resistant to digestion for 30 minutes. This experiment shows that in the intestine of humans or monogastric animals, our engineered protein would likely be more digestible than the wild-type protein would be. These results are consistent with the protease inhibition assays showing that BHL-3N was not an effective protease inhibitor. The inventive proteins were digested in less than five minutes, less than one minute and less than 30 seconds.

Example 6 - Protein Conformation

Wild type CI-2, BHL-1, BHL-2, BHL-3 and BHL-3N at proteins concentrations of approximately 0.16mg/ml in 10mM sodium phosphate, pH = 7.0 were prepared and sent to the University of Michigan Medical School Protein Structure Facility for circular dichroism analysis. Data indicates that the substituted proteins BHL-1, BHL-2 and BHL-3 have very similar CD spectra confirming that the BHL proteins fold into a structure similar to the wild type CI-2.

Example 7 - Thermodynamic stability

Equilibrium denaturation experiments were done to assess the thermodynamic stability of the engineered and wild-type proteins, following the method of Pace et al. (Meth. Enzym. 131:266-280). The engineered or wild-type proteins at a concentration of 2 μ M were incubated 18 hours at 25 degrees centigrade in 10 mM sodium phosphate, pH 7.0, with various concentrations of guanidine-hydrochloride. Unfolding of the proteins was monitored by measuring intrinsic fluorescence at 25 degrees centigrade, using an excitation wavelength of 280 nm and an emission wavelength of 356 nm. The guanidine-hydrochloride concentration sufficient for 50% unfolding was found to be 3.9M for wild-type, 2.4M for BHL-1, and 0.9M for BHL-2, BHL-3, and BHL-3N. These experiments showed that BHL-1 has a higher thermodynamic stability than do the other engineered proteins, but that all of the engineered proteins have a lower thermodynamic stability than does the wild-type protein.

Example 8 - Accessibility of the Tryptophan of BHL Proteins to Acrylamide

Acrylamide effectively quenches the fluorescence of accessible tryptophan residues in proteins. We examined fluorescence quenching of the tryptophan residue of the BHL proteins and of the truncated WT CI-2, in the presence or absence of 6M guanidine-hydrochloride. An excitation wavelength of 295 nm was used. Emission wavelengths of 337 nm and 356 nm were used for the samples without guanidine-HCl and with guanidine-HCl, respectively. Protein concentrations of 20 μ M or 2 μ M were used for the samples without, and with guanidine-HCl, respectively. Samples were in 10 mM sodium phosphate, pH 7.0, and contained acrylamide at the following concentrations: 0, 0.0196M, 0.0385M, 0.0566M, 0.0741M, 0.0909M, 0.1071M, 0.01228M, or 0.1379M. The equation of Mclure and Edelman (Biochem 6: 559-566) was used to correct for self-absorption of light by acrylamide. F_0/F was plotted against the molar acrylamide concentration, where F_0 = fluorescence intensity without acrylamide, and F = fluorescence intensity with acrylamide. The slope of each line (known as the Stern-Volmer constant) was determined. The mean of 2 experiments is presented below. Values in parentheses are standard deviations.

Protein	6M guanidine-HCl	Slope
BHL-1	-	3.5 (0.3)
BHL-1	+	16.9 (1.3)
BHL-2	-	4.6 (0.4)
BHL-2	+	19.0 (0.1)
BHL-3	-	2.4 (0.2)
BHL-3	+	17.5 (0.04)
BHL-3N	-	5.8 (0.1)
BHL-3N	+	16.6 (0.6)
WT CI-2	-	1.7 (0.1)
(truncated)		
WT CI-2	+	15.7(2.1)
(truncated)		

Example 9 - Stabilization by Disulfide Bonds

An examination of the WI-CI 2 three dimensional structure has identified three pairs of residues (Glu-23 and Arg-81, Thr-22 and Val-82, and Val-53 and Val-70) with an alpha carbon distance appropriate for disulfide formation. Constructs designed to substitute these residues with cysteines will be prepared.

NOV 1996

SEQUENCE LISTING

5

(1) GENERAL INFORMATION

- (i) APPLICANT: Pioneer Hi-Bred International, Inc.
- 10 (ii) TITLE OF THE INVENTION: Protein With Enhanced Levels
of Essential Amino Acids
- (iii) NUMBER OF SEQUENCES: 26
- 15 (iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Pioneer Hi-Bred International, Inc.
(B) STREET: 7100 NW 62nd Avenue, P.O. Box 1000
(C) CITY: Johnston
20 (D) STATE: IA
(E) COUNTRY: USA
(F) ZIP: 50131
- 25 (v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Diskette
(B) COMPUTER: IBM Compatible
(C) OPERATING SYSTEM: DOS
30 (D) SOFTWARE: FastSEQ for Windows Version 2.0
- (vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
35 (B) FILING DATE:
(C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: 08/740,682
40 (B) FILING DATE: 01-NOV-1996
- (viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Michel, Marianne H
45 (B) REGISTRATION NUMBER: 35,286
(C) REFERENCE/DOCKET NUMBER: 0571C
- (ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 515-334-4467
50 (B) TELEFAX: 515-334-6883
(C) TELEX:

55

(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 195 base pairs

(B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

5 (ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

(A) NAME/KEY: Coding Sequence

(B) LOCATION: 1...195

10 (D) OTHER INFORMATION:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

15	AAG CTG AAG ACA GAG TGG CCG GAG TTG GTG GGG AAA TCG GTG GAG AAA	48
	Lys Leu Lys Thr Glu Trp Pro Glu Leu Val Gly Lys Ser Val Glu Lys	
	1 5 10 15	
20	GCC AAG AAG GTG ATC CTG AAG GAC AAG CCA GAG GCG CAA ATC ATA GTT	96
	Ala Lys Lys Val Ile Leu Lys Asp Lys Pro Glu Ala Gln Ile Ile Val	
	20 25 30	
25	CTG CCG GTT GGT ACA AAG GTG ACG AAG GAA TAT AAG ATC GAC CGC GTC	144
	Leu Pro Val Gly Thr Lys Val Thr Lys Glu Tyr Lys Ile Asp Arg Val	
	35 40 45	
30	AAG CTC TTT GTG GAT AAA AAG GAC AAC ATC GCG CAG GTC CCC AGG GTC	192
	Lys Leu Phe Val Asp Lys Lys Asp Asn Ile Ala Gln Val Pro Arg Val	
	50 55 60	
35	GGC	195
	Gly	
	65	

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 65 amino acids

40 (B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

45 (v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

50	Lys Leu Lys Thr Glu Trp Pro Glu Leu Val Gly Lys Ser Val Glu Lys
	1 5 10 15
	Ala Lys Lys Val Ile Leu Lys Asp Lys Pro Glu Ala Gln Ile Ile Val
	20 25 30
	Leu Pro Val Gly Thr Lys Val Thr Lys Glu Tyr Lys Ile Asp Arg Val
	35 40 45
55	Lys Leu Phe Val Asp Lys Lys Asp Asn Ile Ala Gln Val Pro Arg Val
	50 55 60
	Gly
	65

400 12 00

(2) INFORMATION FOR SEQ ID NO:3:

5

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 195 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

10

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

15

- (A) NAME/KEY: Coding Sequence
 (B) LOCATION: 1...195
 (D) OTHER INFORMATION:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

20

AAG CTG AAG ACA GAG TGG CCG GAG TTG GTG GGG AAA TCG GTG GAG AAA 48
 Lys Leu Lys Thr Glu Trp Pro Glu Leu Val Gly Lys Ser Val Glu Lys
 1 5 10 15

25

GCC AAG AAG GTG ATC CTG AAG GAC AAG CCA GAG GCG CAA ATC ATA GTT 96
 Ala Lys Lys Val Ile Leu Lys Asp Lys Pro Glu Ala Gln Ile Ile Val
 20 25 30

30

CTA CCG GTT GGT ACA AAG GTG GCG AAG GCC TAT AAG ATC GAC AAG GTC 144
 Leu Pro Val Gly Thr Lys Val Ala Lys Ala Tyr Lys Ile Asp Lys Val
 35 40 45

35

AAG CTT TTT GTG GAT AAA AAG GAC AAC ATC GCG CAG GTC CCC AGG GTC 192
 Lys Leu Phe Val Asp Lys Lys Asp Asn Ile Ala Gln Val Pro Arg Val
 50 55 60

40

GGC 195
 Gly
 65

(2) INFORMATION FOR SEQ ID NO:4:

45

(i) SEQUENCE CHARACTERISTICS:

50

- (A) LENGTH: 65 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(v) FRAGMENT TYPE: internal

55

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Lys Leu Lys Thr Glu Trp Pro Glu Leu Val Gly Lys Ser Val Glu Lys
 1 5 10 15
 Ala Lys Lys Val Ile Leu Lys Asp Lys Pro Glu Ala Gln Ile Ile Val

10

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 195 base pairs
 15 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

20

(A) NAME/KEY: Coding Sequence
(B) LOCATION: 1...195
(D) OTHER INFORMATION:

25

50

55 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 65 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

69

NO. 1000

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

```

5   Lys Leu Lys Thr Glu Trp Pro Glu Leu Val Gly Lys Ser Val Glu Lys
    1           5           10           15
    Ala Lys Lys Val Ile Leu Lys Asp Lys Pro Glu Ala Gln Ile Ile Val
      20           25           30
    Leu Pro Val Gly Thr Lys Val Gly Lys His Tyr Lys Ile Asp Lys Val
10   35           40           45
    Lys Leu Phe Val Asp Lys Lys Asp Asn Ile Ala Gln Val Pro Arg Val
      50           55           60
    Gly
    65
15

```

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60 61 62 63 64 65 66 67 68 69 70 71 72 73 74 75 76 77 78 79 80 81 82 83 84 85 86 87 88 89 90 91 92 93 94 95 96 97 98 99 100

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

- 5 (A) LENGTH: 249 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

- 10 (A) NAME/KEY: Coding Sequence
(B) LOCATION: 1...249
(D) OTHER INFORMATION:

15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

20 AAG TCG GTG GAG AAG AAA CCG AAG GGT GTG AAG ACA GGT GCG GGT GAC 48
Lys Ser Val Glu Lys Lys Pro Lys Gly Val Lys Thr Gly Ala Gly Asp
1 5 10 15

AAG CAT AAG CTG AAG ACA GAG TGG CCG GAG TTG GTG GGG AAA TCG GTG 96
Lys His Lys Leu Lys Thr Glu Trp Pro Glu Leu Val Gly Lys Ser Val
20 25 30

25 GAG AAA GCC AAG AAG GTG ATC CTG AAG GAC AAG CCA GAG GCG CAA ATC 144
Glu Lys Ala Lys Lys Val Ile Leu Lys Asp Lys Pro Glu Ala Gln Ile
35 40 45

30 ATA GTT CTA CCG GTT GGT ACA AAG GTG GGT AAG CAT TAT AAG ATC GAC 192
Ile Val Leu Pro Val Gly Thr Lys Val Gly Lys His Tyr Lys Ile Asp
50 55 60

35 AAG GTC AAG CTT TTT GTG GAT AAA AAG GAC AAC ATC GCG CAG GTC CCC 240
Lys Val Lys Leu Phe Val Asp Lys Lys Asp Asn Ile Ala Gln Val Pro
65 70 75 80

40 AGG GTC GGC 249
Arg Val Gly

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

- 45 (A) LENGTH: 83 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

50

(ii) MOLECULE TYPE: protein

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

55

Lys Ser Val Glu Lys Lys Pro Lys Gly Val Lys Thr Gly Ala Gly Asp
1 5 10 15
Lys His Lys Leu Lys Thr Glu Trp Pro Glu Leu Val Gly Lys Ser Val

10

15

20

(ix) FEATURE:

25

(D) OTHER INFORMATION:

30

35

40

45

50

AGG GTC GGC 249
Arg Val Gly

55

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 83 amino acids

14.00.10.00

- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

5

- (ii) MOLECULE TYPE: protein
- (v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

[illegible]

15

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

- 20 (A) LENGTH: 249 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

25 (ix) FEATURE:

- (A) NAME/KEY: Coding Sequence
(B) LOCATION: 1...249
(D) OTHER INFORMATION:

30

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

35	AAG TCG GTG GAG AAG AAA CCG AAG GGT GTG AAG ACA GGT GCG GGT GAC Lys Ser Val Glu Lys Lys Pro Lys Gly Val Lys Thr Gly Ala Gly Asp 1 5 10 15	48
40	AAG CAT AAG CTG AAG ACA GAG TGG CCG GAG TTG GTG GGG AAA TCG GTG Lys His Lys Leu Lys Thr Glu Trp Pro Glu Leu Val Gly Lys Ser Val 20 25 30	96
45	GAG AAA GCC AAG AAG GTG ATC CTG AAG GAC AAG CCA GAG GCG CAA ATC Glu Lys Ala Lys Lys Val Ile Leu Lys Asp Lys Pro Glu Ala Gln Ile 35 40 45	144
50	ATA GTT CTA CCG GTT GGT ACA AAG GTG GCG AAG GCC TAT AAG ATC GAC Ile Val Leu Pro Val Gly Thr Lys Val Ala Lys Ala Tyr Lys Ile Asp 50 55 60	192
50	AAG GTC AAG CTT TTT GTG GAT AAA AAG GAC AAC ATC GCG CAG GTC CCC Lys Val Lys Leu Phe Val Asp Lys Lys Asp Asn Ile Ala Gln Val Pro 65 70 75 80	240
	AGG GTC GGC Arg Val Gly	249

(2) INFORMATION FOR SEQ ID NO:12:

NO. 12. 00

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 83 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

Lys Ser Val Glu Lys Lys Pro Lys Gly Val Lys Thr Gly Ala Gly Asp
 1 5 10 15
 Lys His Lys Leu Lys Thr Glu Trp Pro Glu Leu Val Gly Lys Ser Val
 20 25 30
 Glu Lys Ala Lys Lys Val Ile Leu Lys Asp Lys Pro Glu Ala Gln Ile
 35 40 45
 Ile Val Leu Pro Val Gly Thr Lys Val Ala Lys Ala Tyr Lys Ile Asp
 50 55 60
 Lys Val Lys Leu Phe Val Asp Lys Lys Asp Asn Ile Ala Gln Val Pro
 65 70 75 80
 Arg Val Gly

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 249 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

- (A) NAME/KEY: Coding Sequence
 (B) LOCATION: 1...249
 (D) OTHER INFORMATION:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

AGT TCA GTG GAG AAG AAG CCG GAG GGA GTG AAC ACC GGT GCT GGT GAC 48
 45 Ser Ser Val Glu Lys Lys Pro Glu Gly Val Asn Thr Gly Ala Gly Asp
 1 5 10 15
 CGT CAC AAC CTG AAG ACA GAG TGG CCA GAG TTG GTG GGG AAA TCG GTG 96
 50 Arg His Asn Leu Lys Thr Glu Trp Pro Glu Leu Val Gly Lys Ser Val
 20 25 30
 GAG GAG GCC AAG AAG GTG ATT CTG CAG GAC AAG CCA GAG GCG CAA ATC 144
 55 Glu Glu Ala Lys Lys Val Ile Leu Gln Asp Lys Pro Glu Ala Gln Ile
 35 40 45
 ATA GTT CTA CCG GTG GGG ACA ATT GTG ACC ATG GAA TAT CGG ATC GAC 192
 Ile Val Leu Pro Val Gly Thr Ile Val Thr Met Glu Tyr Arg Ile Asp
 50 55 60

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60 61 62 63 64 65 66 67 68 69 70 71 72 73 74 75 76 77 78 79 80 81 82 83 84 85 86 87 88 89 90 91 92 93 94 95 96 97 98 99 100

CGC GTC CGC CTC TTT GTC GAT AAA CTC GAC AAC ATT GCC CAG GTC CCC 240
 Arg Val Arg Leu Phe Val Asp Lys Leu Asp Asn Ile Ala Gln Val Pro
 65 70 75 80

5

AGG GTC GGC 249
 Arg Val Gly

10

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

15

- (A) LENGTH: 83 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

20

- (ii) MOLECULE TYPE: protein
- (v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

25 Ser Ser Val Glu Lys Lys Pro Glu Gly Val Asn Thr Gly Ala Gly Asp
 1 5 10 15
 Arg His Asn Leu Lys Thr Glu Trp Pro Glu Leu Val Gly Lys Ser Val
 20 25 30
 Glu Glu Ala Lys Lys Val Ile Leu Gln Asp Lys Pro Glu Ala Gln Ile
 35 40 45
 30 Ile Val Leu Pro Val Gly Thr Ile Val Thr Met Glu Tyr Arg Ile Asp
 50 55 60
 Arg Val Arg Leu Phe Val Asp Lys Leu Asp Asn Ile Ala Gln Val Pro
 65 70 75 80
 Arg Val Gly

35

(2) INFORMATION FOR SEQ ID NO:15:

40

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 459 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

45

- (ii) MOLECULE TYPE: cDNA
- (ix) FEATURE:

50

- (A) NAME/KEY: Coding Sequence
- (B) LOCATION: 1...288
- (D) OTHER INFORMATION:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

55 GCA GTG CAA CAA GCA AGA TTT ACC TGC CCA TCG ATC ATA TCG TCA ACT 48
 Ala Val Gln Gln Ala Arg Phe Thr Cys Pro Ser Ile Ile Ser Ser Thr
 1 5 10 15

25

30

(A) LENGTH: 96 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

35

(xi) SEQUENCE DESCRIPTION: SEO ID NO:16:

55

(A) LENGTH: 428 base pairs

.

(B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

5 (ii) MOLECULE TYPE: cDNA
 (ix) FEATURE:

(A) NAME/KEY: Coding Sequence

(B) LOCATION: 1...303

10 (D) OTHER INFORMATION:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

15	CGA CCC ACG CGT CCG CCC ACG CGT CCG GCA AGA TTT ACC TGC CCA TCG	48
	Arg Pro Thr Arg Pro Pro Thr Arg Pro Ala Arg Phe Thr Cys Pro Ser	
	1 5 10 15	
20	ATC ATA TCG TCA ACT GGT CCG GCA GTT CGC GAC ACC ATG AGC TCC ACG	96
	Ile Ile Ser Ser Thr Gly Pro Ala Val Arg Asp Thr Met Ser Ser Thr	
	20 25 30	
25	GAG TGC GGC GGC GGC GGC GGC GGC GCC AAG ACG TCG TGG CCT GAG GTG	144
	Glu Cys Gly Gly Gly Gly Gly Gly Ala Lys Thr Ser Trp Pro Glu Val	
	35 40 45	
	GTC GGG CTG AGC GTG GAG GAC GCC AAG AAG GTG ATC CTC AAG GAC AAG	192
	Val Gly Leu Ser Val Glu Asp Ala Lys Lys Val Ile Leu Lys Asp Lys	
	50 55 60	
30	CCG GAC GCC GAC ATC GTG GTG CTG CCC GTC GGC TCC GTG GTG ACC GCG	240
	Pro Asp Ala Asp Ile Val Val Leu Pro Val Gly Ser Val Val Thr Ala	
	65 70 75 80	
35	GAT TAT CGC CCT AAC CGT GTC CGC ATC TTC GTC GAC ATC GTC GCC CAG	288
	Asp Tyr Arg Pro Asn Arg Val Arg Ile Phe Val Asp Ile Val Ala Gln	
	85 90 95	
40	ACG CCC CAC ATC GGC TGATAATATA TAAGCTAGCC GCTATTTCCT TTCCTTGCCC C	344
	Thr Pro His Ile Gly	
	100	
	AGAACTTGAA ATAAATATAT ATACGATGAA ATAACGCGGG CATGCCGAAT AATGGATGTG	404
	TGAAAAAAAAA AAAAAAAAAA AAAA	428

45

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:

50 (A) LENGTH: 101 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

55 (ii) MOLECULE TYPE: protein
 (v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

0571R-PCT

.....

Arg Pro Thr Arg Pro Pro Thr Arg Pro Ala Arg Phe Thr Cys Pro Ser
 1 5 10 15
 Ile Ile Ser Ser Thr Gly Pro Ala Val Arg Asp Thr Met Ser Ser Thr
 20 25 30
 Glu Cys Gly Gly Gly Gly Gly Gly Ala Lys Thr Ser Trp Pro Glu Val
 35 40 45
 Val Gly Leu Ser Val Glu Asp Ala Lys Lys Val Ile Leu Lys Asp Lys
 50 55 60
 Pro Asp Ala Asp Ile Val Val Leu Pro Val Gly Ser Val Val Thr Ala
 65 70 75 80
 Asp Tyr Arg Pro Asn Arg Val Arg Ile Phe Val Asp Ile Val Ala Gln
 85 90 95
 Thr Pro His Ile Gly
 100

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 441 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

- (A) NAME/KEY: Coding Sequence
 (B) LOCATION: 1...255
 (D) OTHER INFORMATION:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

TTA ATT ATT GCC CTT TCA GTT NGC CAT CGG CAG CCG AGC ACC ATG AGC 48
 Leu Ile Ile Ala Leu Ser Val Xaa His Arg Gln Pro Ser Thr Met Ser
 1 5 10 15
 TCC ACA GGC GGC GGC GAC GAT GGC GCC AAG AAG TCT TGG CCG GAA GTG 96
 Ser Thr Gly Gly Gly Asp Asp Gly Ala Lys Lys Ser Trp Pro Glu Val
 20 25 30
 GTC GGG CTC AGC CTG GAA GAA GCC AAG AGG GTG ATC CTG TGC GAC AAG 144
 Val Gly Leu Ser Leu Glu Glu Ala Lys Arg Val Ile Leu Cys Asp Lys
 35 40 45
 CCC GAC GCC GAC ATC GTC GTG CTG CCC GTC GGC ACG CCG GTG ACC ATG 192
 Pro Asp Ala Asp Ile Val Val Leu Pro Val Gly Thr Pro Val Thr Met
 50 55 60
 GAT TTC CGC CCC AAC CGC GTC CGC ATC TTC GTC GAC ACC GTC GCG GAG 240
 Asp Phe Arg Pro Asn Arg Val Arg Ile Phe Val Asp Thr Val Ala Glu
 65 70 75 80
 GCA MCC CAC ATC GGC TGAGGTAAA TCTACAAAT GAATGAYTCG GACATGCCAT G 296
 Ala Xaa His Ile Gly
 85

INSTRUMENT

NO. 1000

CGTACNTGTC CGTCGCCGAA TAATGGATGT GTGTGTGCTT CGATCGTTCC TAATAAGTTG 356
CTAGTNAAAA ATAATNGGCA TCGTCGTTAN TGCATGAATA AAAAGTATCA GAATAATGTT 416
CACCCTTTTCN AAAAAAAAAA AAAAA 441

5

(2) INFORMATION FOR SEQ ID NO:20:

10

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 85 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

15

(ii) MOLECULE TYPE: protein

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

20

Leu Ile Ile Ala Leu Ser Val Xaa His Arg Gln Pro Ser Thr Met Ser
1 5 10 15
Ser Thr Gly Gly Gly Asp Asp Gly Ala Lys Lys Ser Trp Pro Glu Val
20 25 30
Val Gly Leu Ser Leu Glu Glu Ala Lys Arg Val Ile Leu Cys Asp Lys
25 35 40 45
Pro Asp Ala Asp Ile Val Val Leu Pro Val Gly Thr Pro Val Thr Met
50 55 60
Asp Phe Arg Pro Asn Arg Val Arg Ile Phe Val Asp Thr Val Ala Glu
30 65 70 75 80
Ala Xaa His Ile Gly
85

35

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 382 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

40

(ii) MOLECULE TYPE: cDNA

45

(ix) FEATURE:

- (A) NAME/KEY: Coding Sequence
(B) LOCATION: 1...213
(D) OTHER INFORMATION:

50

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

GTG CGT CGT CGG CGA ACA GCC ACC GGC GGC AAG ACG TCG TGG CCG GAG 48
Val Arg Arg Arg Arg Thr Ala Thr Gly Gly Lys Thr Ser Trp Pro Glu
55 1 5 10 15
GTG GTC GGG CTG AGC GTC GAG GAA GCC AAG AAG GTG ATT CTG GCG GAC 96
Val Val Gly Leu Ser Val Glu Glu Ala Lys Lys Val Ile Leu Ala Asp

20

25 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 71 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

30 (v) FRAGMENT TYPE: internal

[illegible]

50 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 448 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

55 (ii) MOLECULE TYPE: cDNA
(ix) FEATURE:

81

AMENDED SPEC

11 00 10 00

(B) LOCATION: 1...240

(D) OTHER INFORMATION:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

5 CGA TTT AGC TAT AGC AGG TCT CGA TCG GCG GCC ATG AGC GGT AGC CGC 48
Arg Phe Ser Tyr Ser Arg Ser Arg Ser Ala Ala Met Ser Gly Ser Arg
1 5 10 15

10 AGC AAG AAG TCG TGG CCG GAG GTG GAG GGG CTG CCG TCC GAG GTG GCC 96
Ser Lys Lys Ser Trp Pro Glu Val Glu Gly Leu Pro Ser Glu Val Ala
20 25 30

15 AAG CAG AAA ATT CTG GCC GAC CGC CCG GAC GTC CAG GTG GTC GTT CTG 144
Lys Gln Lys Ile Leu Ala Asp Arg Pro Asp Val Gln Val Val Val Leu
35 40 45

20 CCC GAC GGC TCC TTC GTC ACC ACT GAT TTC AAC GAC AAG CGC GTC CGG 192
Pro Asp Gly Ser Phe Val Thr Thr Asp Phe Asn Asp Lys Arg Val Arg
50 55 60

25 GTC TTC GTC GAC AAC GCC GAC AAC GTC GCC AAA GTC CCC AAG ATC GGC T 241
Val Phe Val Asp Asn Ala Asp Asn Val Ala Lys Val Pro Lys Ile Gly
65 70 75 80

30 AGCTAGCTAG CTAGGCCCAA TCGTTCTAAT CAGCTAGTTT CTTTCTTTCA TAAATAAAAG 301
TCCTCTCTCG TACCCGGACT GTGATGTTTC CCTAGTTGTC TCGTACGTGT TGTTTTCTGT 361
CTTAATGGAT GCCATGGCGC CCGCGCGCGC CTYCATCATG AAAAGCTACA TTTGAAACGA 421
TTTTNAGTAT TCTTTGCTGT TAAAAAA 448

(2) INFORMATION FOR SEQ ID NO:24:

35 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 80 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

40 (ii) MOLECULE TYPE: protein
(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

45 Arg Phe Ser Tyr Ser Arg Ser Arg Ser Ala Ala Met Ser Gly Ser Arg
1 5 10 15
Ser Lys Lys Ser Trp Pro Glu Val Glu Gly Leu Pro Ser Glu Val Ala
20 25 30

50 Lys Gln Lys Ile Leu Ala Asp Arg Pro Asp Val Gln Val Val Val Leu
35 40 45
Pro Asp Gly Ser Phe Val Thr Asp Phe Asn Asp Lys Arg Val Arg
50 55 60
Val Phe Val Asp Asn Ala Asp Asn Val Ala Lys Val Pro Lys Ile Gly
55 65 70 75 80

5 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

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20 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

18

11 00 10 00

- All publications and patent applications mentioned in this specification are indicative of the level of skill of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and
- 5 individually indicated to be incorporated by reference.

Variations on the above embodiments are within the ability of one of ordinary skill in the art, and such variations do not depart from the scope of the present invention as described in the following claims.